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(54) **METHODS FOR IDENTIFYING A TARGET SITE OF A CAS9 NUCLEASE**

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(58) **Field of Classification Search**
None
See application file for complete search history.

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(57) **ABSTRACT**

Some aspects of this disclosure provide strategies, methods, and reagents for determining nuclease target site preferences and specificity of site-specific endonucleases. Some methods provided herein utilize a novel "one-cut" strategy for screening a library of concatemers comprising repeat units of candidate nuclease target sites and constant insert regions to identify library members that can been cut by a nuclease of interest via sequencing of an intact target site adjacent and identical to a cut target site.

24 Claims, 55 Drawing Sheets

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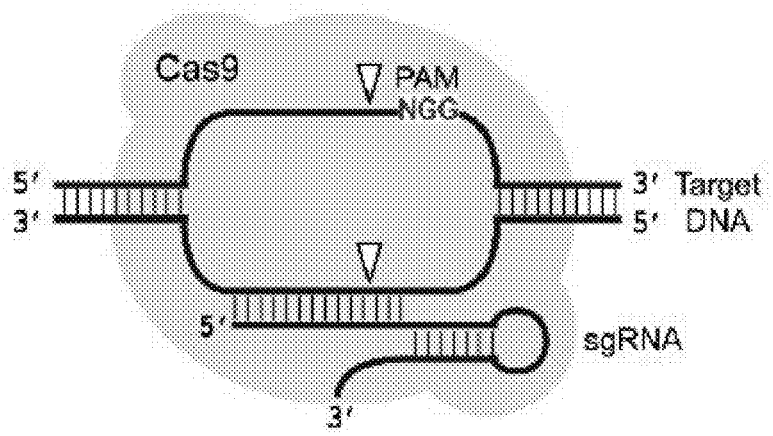


FIG. 1A

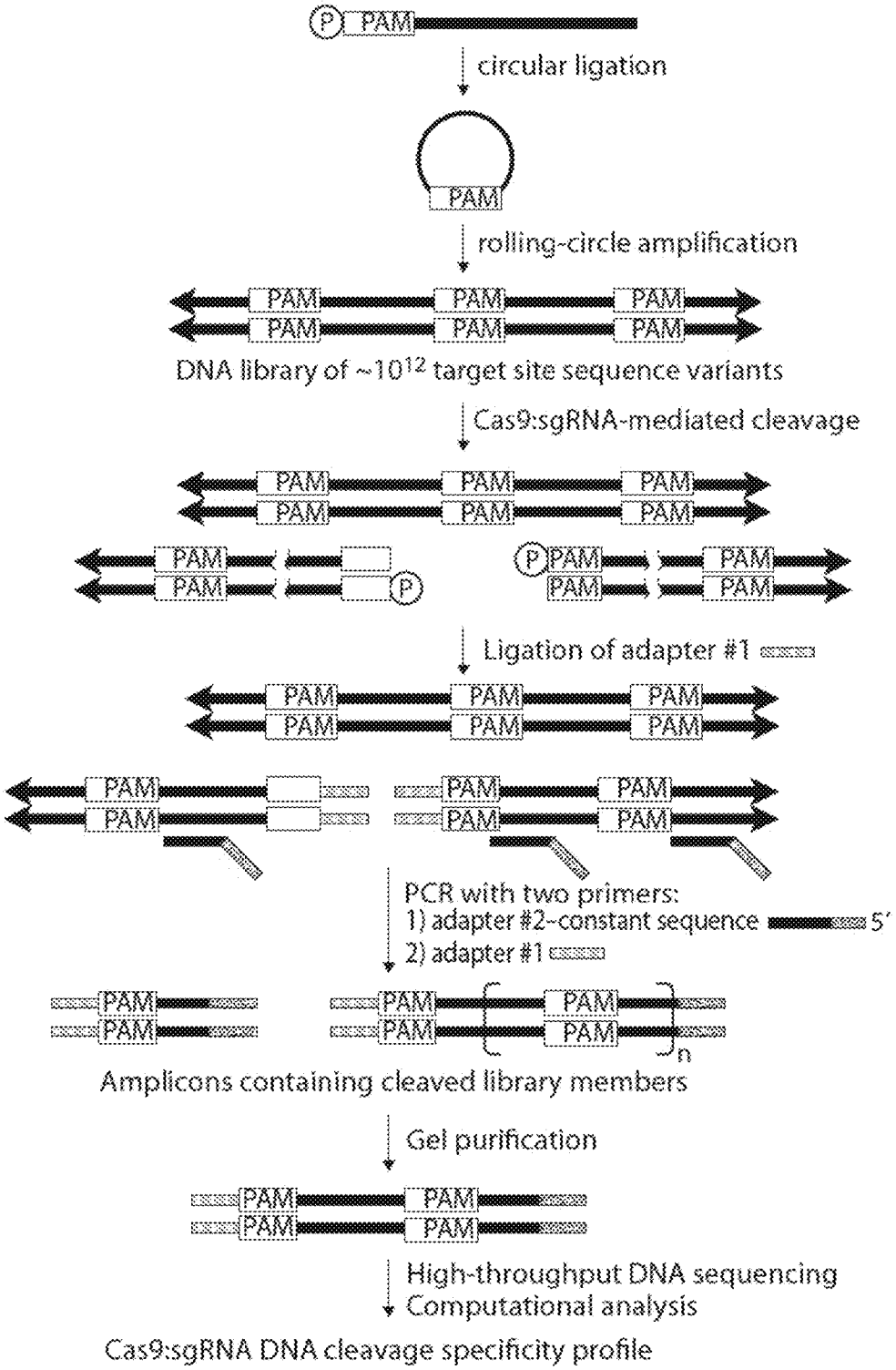
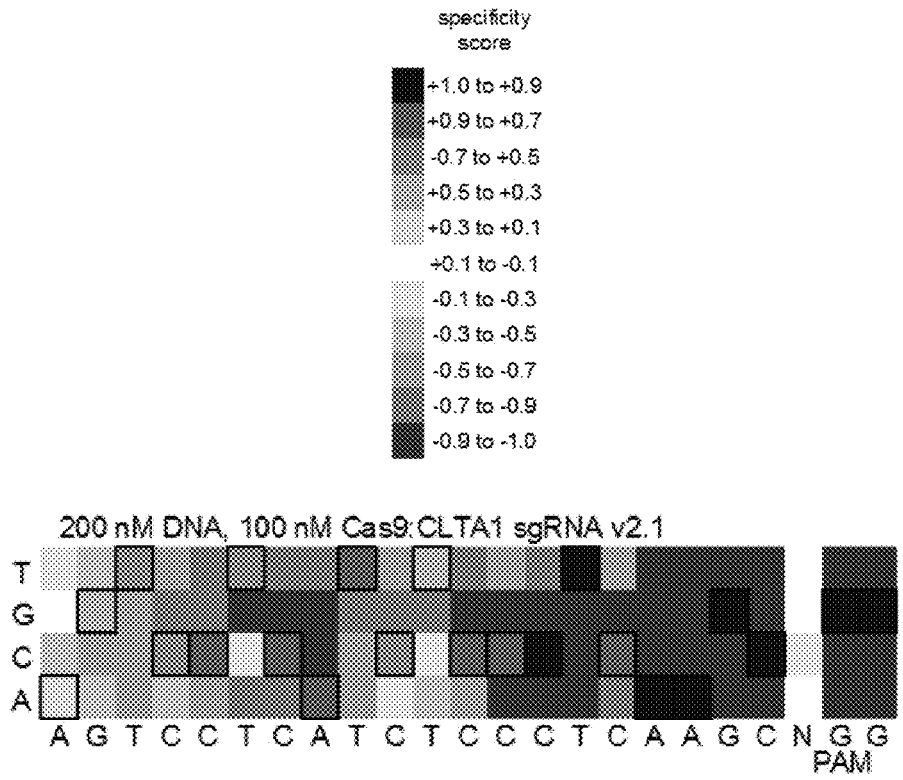

PAM
circular ligation
PAM
rolling-circle amplification
DNA library of ~10^12 target site sequence variants
Cas9:sgRNA-mediated cleavage
Ligation of adapter #1
PCR with two primers:
1) adapter #2-constant sequence 5′
2) adapter #1
n
Amplicons containing cleaved library members
Gel purification
High-throughput DNA sequencing
Computational analysis
Cas9:sgRNA DNA cleavage specificity profile

FIG. 1B


specificity score
+1.0 to +0.9
+0.9 to +0.7
-0.7 to +0.5
+0.5 to +0.3
+0.3 to +0.1
+0.1 to -0.1
-0.1 to -0.3
-0.3 to -0.5
-0.5 to -0.7
-0.7 to -0.9
-0.9 to -1.0
200 nM DNA, 100 nM Cas9:CLTA1 sgRNA v2.1
T
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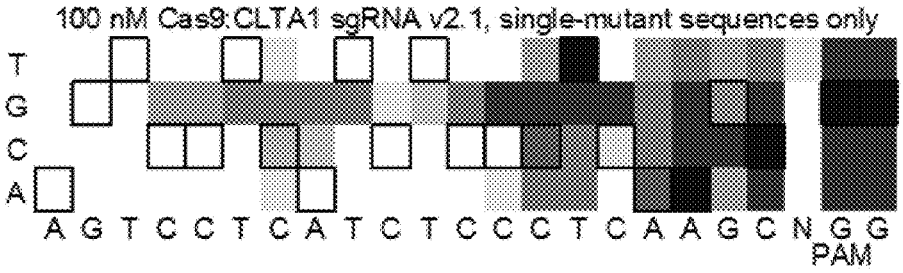
A G T C C T C A T C T C C C T C A A G C N G G
PAM

FIG. 2A


100 nM Cas9:CLTA1 sgRNA v2.1, single-mutant sequences only
T
G
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A G T C C T C A T C T C C C T C A A G C N G G
PAM

FIG. 2B

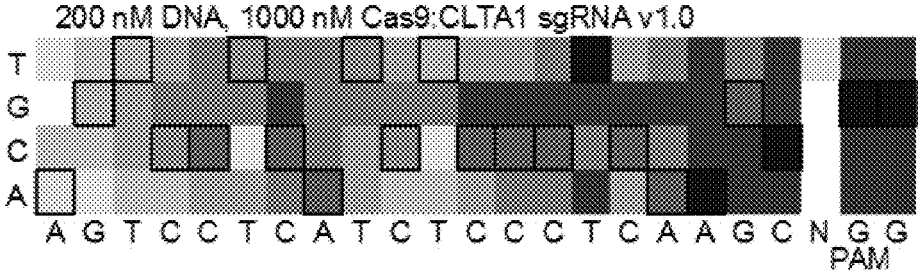


FIG. 2C

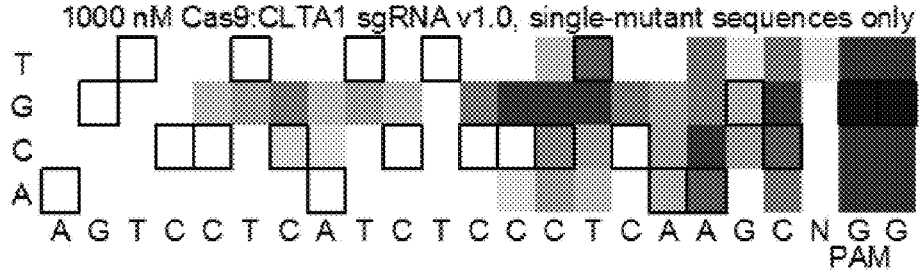


FIG. 2D

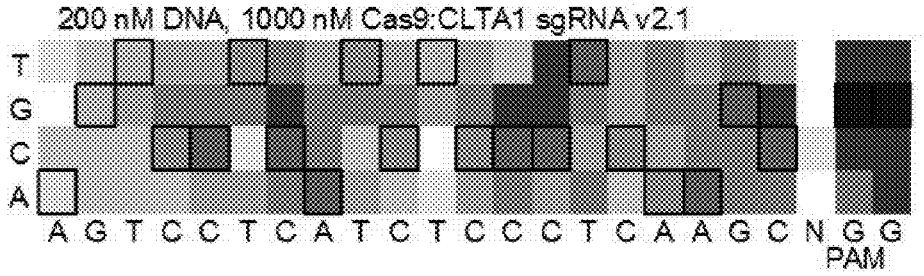


FIG. 2E

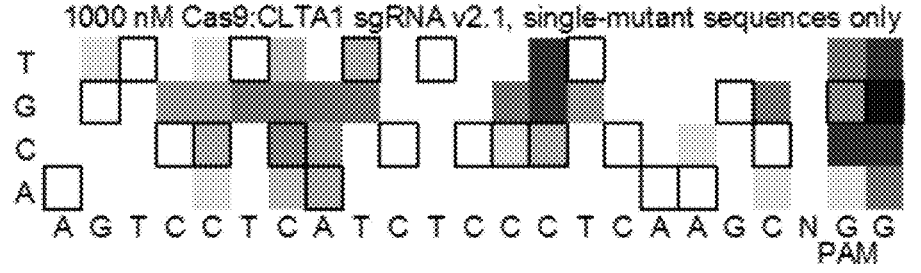


FIG. 2F

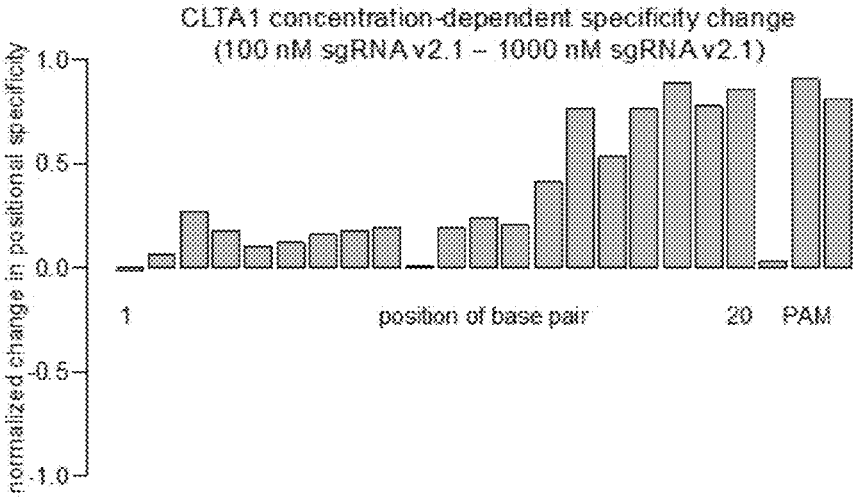

CLTA1 concentration-dependent specificity change
(100 nM sgRNA v2.1 – 1000 nM sgRNA v2.1)
normalized change in positional specificity
1.0
0.5
0.0
-0.5
-1.0
1
position of base pair
20
PAM

FIG. 2G

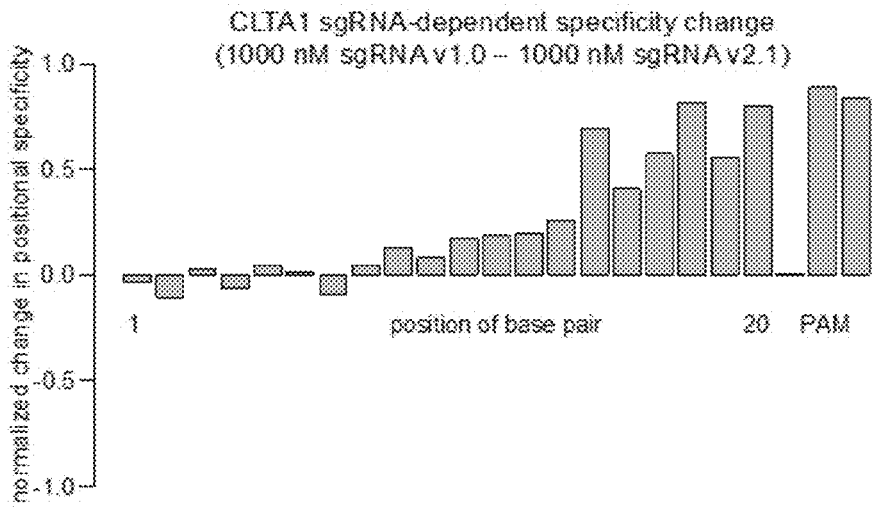

CLTA1 sgRNA-dependent specificity change
(1000 nM sgRNA v1.0 – 1000 nM sgRNA v2.1)
normalized change in positional specificity
1.0
0.5
0.0
-0.5
-1.0
1
position of base pair
20
PAM

FIG. 2H

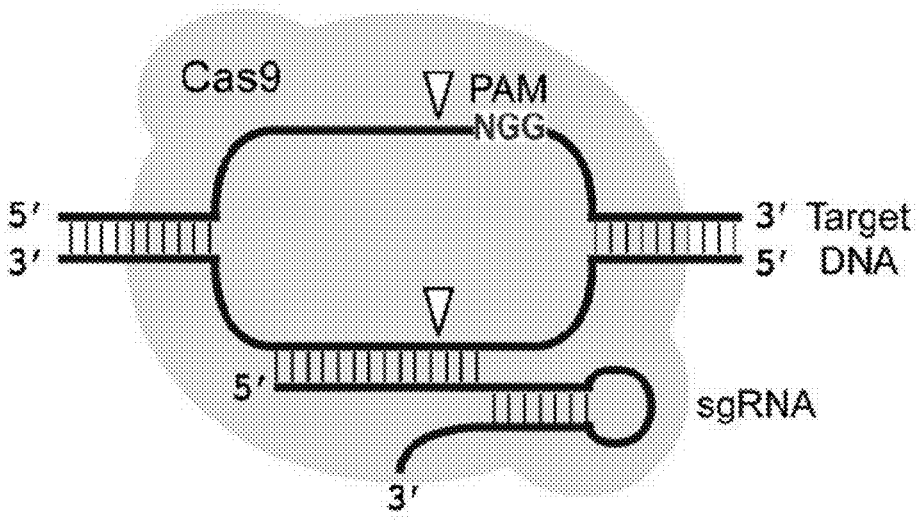

Cas9
PAM
NGG
5′
3′
3′ Target
5′ DNA
5′
sgRNA
3′

FIG. 3A

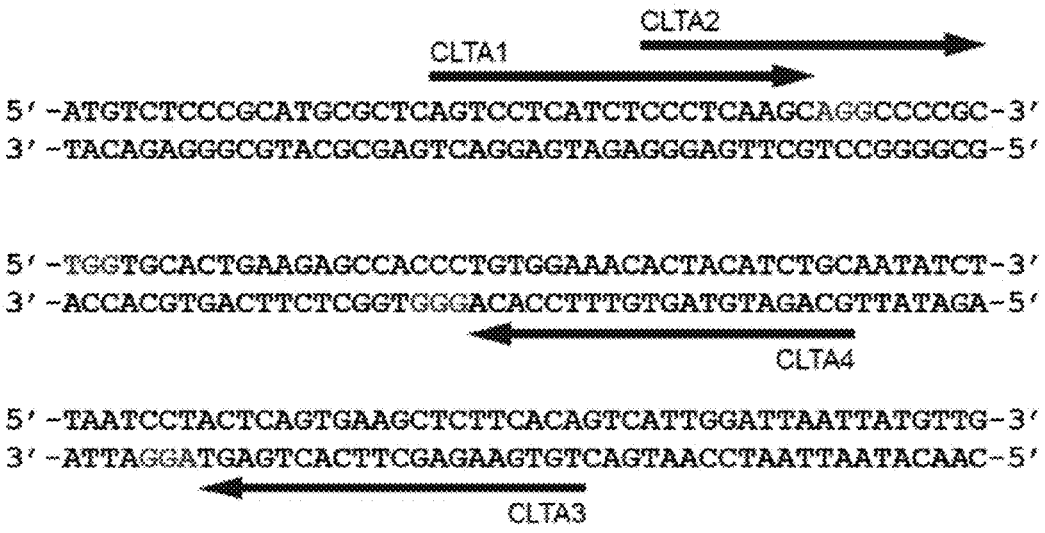

CLTA2
CLTA1
5′-ATGTCTCCCGCATGCGCTCAGTCCTCATCTCCCTCAAGCAGGCCCCGC-3′
3′-TACAGAGGGCGTACGCGAGTCAGGAGTAGAGGGAGTTCGTCCGGGGCG-5′
5′-TGGTGCACTGAAGAGCCACCCTGTGGAAACACTACATCTGCAATATCT-3′
3′-ACCACGTGACTTCTCGGTGGGACACCTTTGTGATGTAGACGTTATAGA-5′
CLTA4
5′-TAATCCTACTCAGTGAAGCTCTTCACAGTCATTGGATTAATTATGTTG-3′
3′-ATTAGGATGAGTCACTTCGAGAAGTGTCAGTAACCTAATTAATACAAC-5′
CLTA3

FIG. 3B

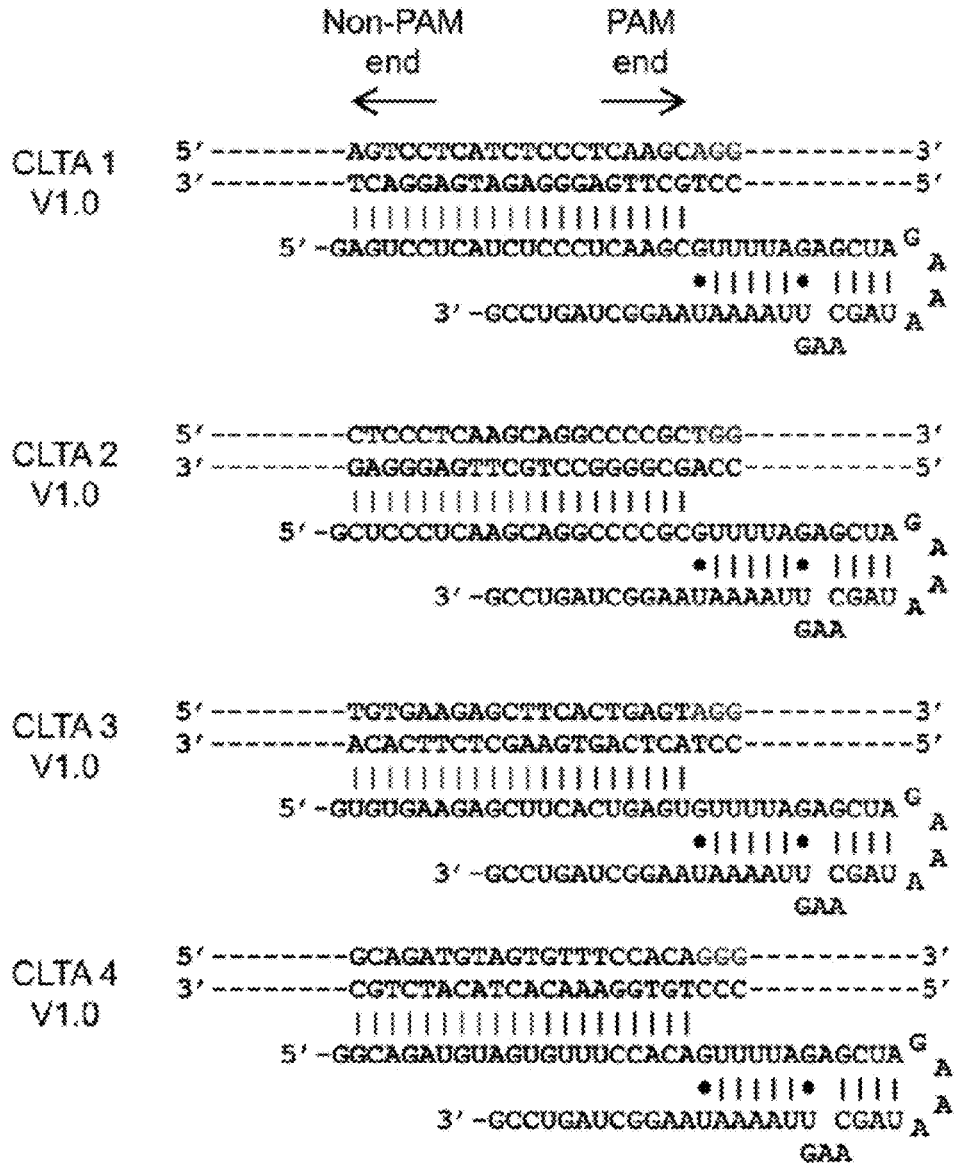

Non-PAM end
PAM end
CLTA 1 V1.0
5′--------AGTCCTCATCTCCCTCAAGCAGG----------3′
3′--------TCAGGAGTAGAGGGAGTTCGTCC----------5′
5′-GAGUCCUCAUCUCCCUCAAGCGUUUUAGAGCUA G A
3′-GCCUGAUCGGAAUAAAAUU CGAU A A
GAA
CLTA 2 V1.0
5′--------CTCCCTCAAGCAGGCCCCGCTGG----------3′
3′--------GAGGGAGTTCGTCCGGGGCGACC----------5′
5′-GCUCCCUCAAGCAGGCCCCGCGUUUUAGAGCUA G A
3′-GCCUGAUCGGAAUAAAAUU CGAU A A
GAA
CLTA 3 V1.0
5′--------TGTGAAGAGCTTCACTGAGTAGG----------3′
3′--------ACACTTCTCGAAGTGACTCATCC----------5′
5′-GUGUGAAGAGCUUCACUGAGUGUUUUAGAGCUA G A
3′-GCCUGAUCGGAAUAAAAUU CGAU A A
GAA
CLTA 4 V1.0
5′--------GCAGATGTAGTGTTTCCACAGGG----------3′
3′--------CGTCTACATCACAAAGGTGTCCC----------5′
5′-GGCAGAUGUAGUGUUUCCACAGUUUUAGAGCUA G A
3′-GCCUGAUCGGAAUAAAAUU CGAU A A
GAA

FIG. 3C

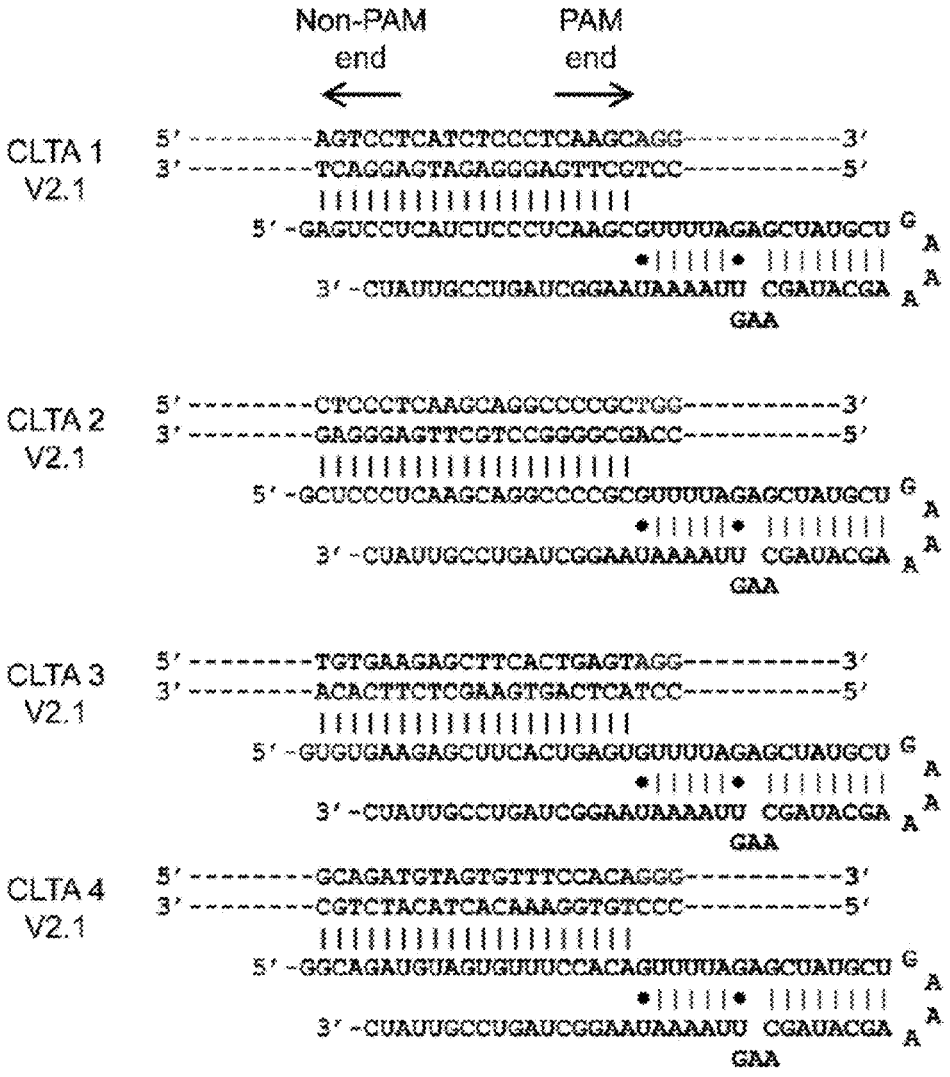

Non-PAM end
PAM end
CLTA 1 V2.1
5′ ---------AGTCCTCATCTCCCTCAAGCAGG----------3′
3′ ---------TCAGGAGTAGAGGGAGTTCGTCC----------5′
5′ -GAGUCCUCAUCUCCCUCAAGCGUUUUAGAGCUAUGCU
3′ -CUAUUGCCUGAUCGGAAUAAAAUU CGAUACGA
GAA
CLTA 2 V2.1
5′ ---------CTCCCTCAAGCAGGCCCCGCTGG----------3′
3′ ---------GAGGGAGTTCGTCCGGGGCGACC----------5′
5′ -GCUCCCUCAAGCAGGCCCCGCGUUUUAGAGCUAUGCU
3′ -CUAUUGCCUGAUCGGAAUAAAAUU CGAUACGA
GAA
CLTA 3 V2.1
5′ ---------TGTGAAGAGCTTCACTGAGTAGG----------3′
3′ ---------ACACTTCTCGAAGTGACTCATCC----------5′
5′ -GUGUGAAGAGCUUCACUGAGUGUUUUAGAGCUAUGCU
3′ -CUAUUGCCUGAUCGGAAUAAAAUU CGAUACGA
GAA
CLTA 4 V2.1
5′ ---------GCAGATGTAGTGTTTCCACAGGG----------3′
3′ ---------CGTCTACATCACAAAGGTGTCCC----------5′
5′ -GGCAGAUGUAGUGUUUCCACAGUUUUAGAGCUAUGCU
3′ -CUAUUGCCUGAUCGGAAUAAAAUU CGAUACGA
GAA

FIG. 3D

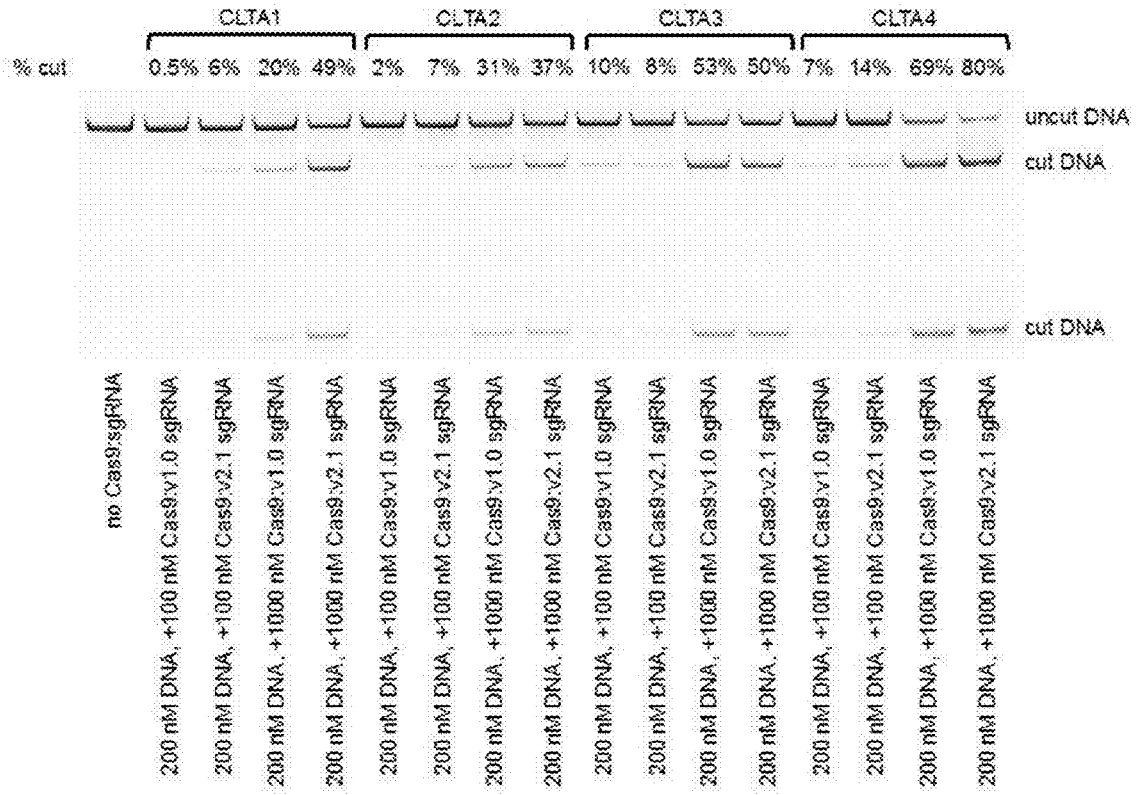

CLTA1
CLTA2
CLTA3
CLTA4
% cut
0.5% 6% 20% 49% 2% 7% 31% 37% 10% 8% 53% 50% 7% 14% 69% 80%
uncut DNA
cut DNA
cut DNA
no Cas9:sgRNA
200 nM DNA, +100 nM Cas9:v1.0 sgRNA
200 nM DNA, +100 nM Cas9:v2.1 sgRNA
200 nM DNA, +1000 nM Cas9:v1.0 sgRNA
200 nM DNA, +1000 nM Cas9:v2.1 sgRNA

FIG. 4

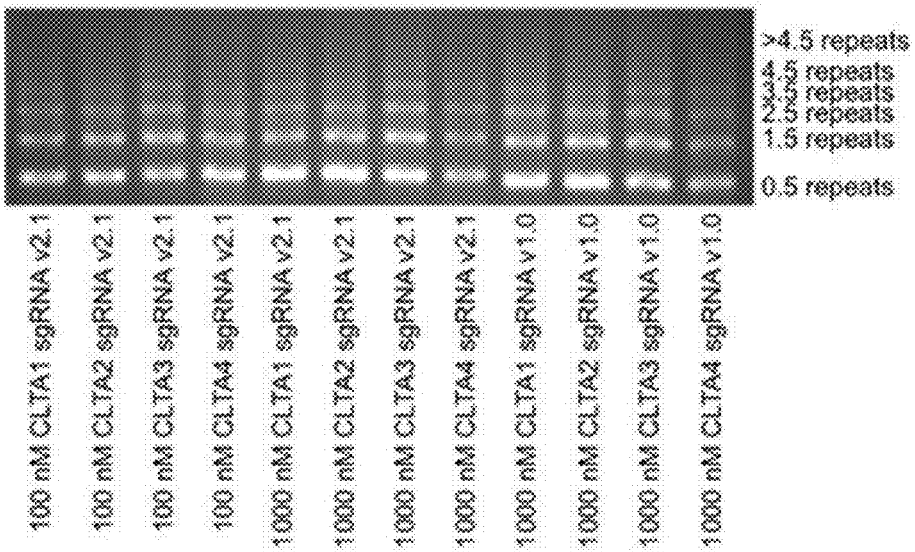

>4.5 repeats
4.5 repeats
3.5 repeats
2.5 repeats
1.5 repeats
0.5 repeats
100 nM CLTA1 sgRNA v2.1
100 nM CLTA2 sgRNA v2.1
100 nM CLTA3 sgRNA v2.1
100 nM CLTA4 sgRNA v2.1
1000 nM CLTA1 sgRNA v2.1
1000 nM CLTA2 sgRNA v2.1
1000 nM CLTA3 sgRNA v2.1
1000 nM CLTA4 sgRNA v2.1
1000 nM CLTA1 sgRNA v1.0
1000 nM CLTA2 sgRNA v1.0
1000 nM CLTA3 sgRNA v1.0
1000 nM CLTA4 sgRNA v1.0

FIG. 5A

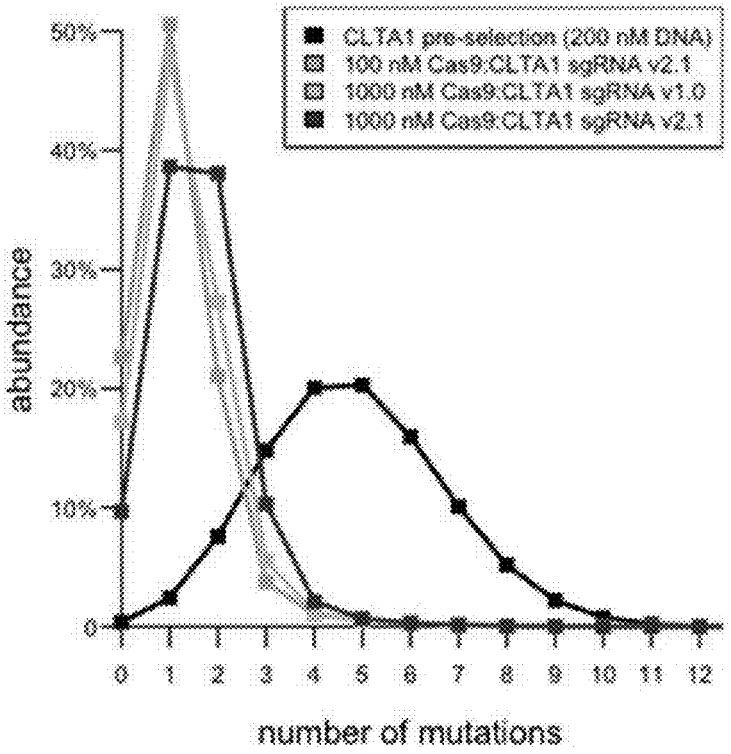

CLTA1 pre-selection (200 nM DNA)
100 nM Cas9:CLTA1 sgRNA v2.1
1000 nM Cas9:CLTA1 sgRNA v1.0
1000 nM Cas9:CLTA1 sgRNA v2.1
50%
40%
30%
20%
10%
0
abundance
0 1 2 3 4 5 6 7 8 9 10 11 12
number of mutations

FIG. 5B

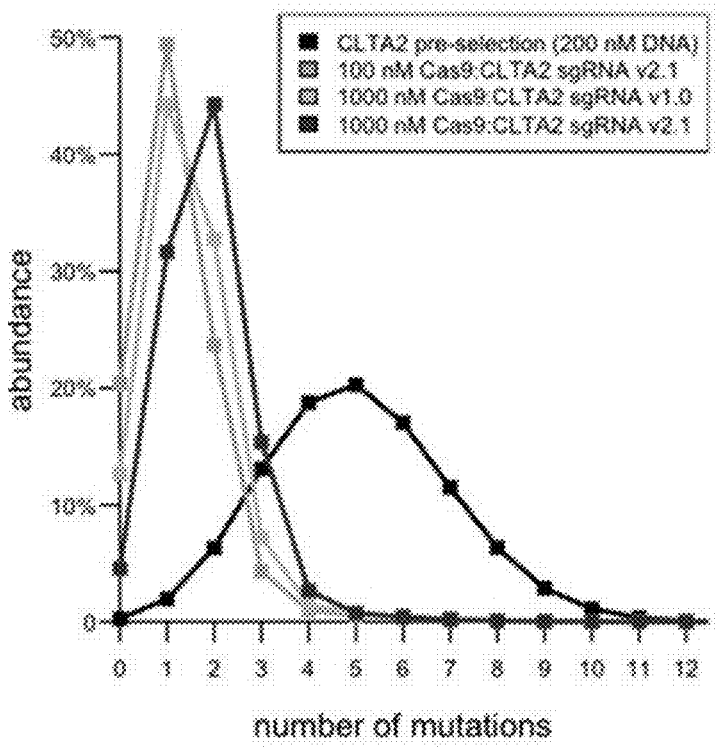

CLTA2 pre-selection (200 nM DNA)
100 nM Cas9:CLTA2 sgRNA v2.1
1000 nM Cas9:CLTA2 sgRNA v1.0
1000 nM Cas9:CLTA2 sgRNA v2.1
50%
40%
30%
20%
10%
0
abundance
0 1 2 3 4 5 6 7 8 9 10 11 12
number of mutations

FIG. 5C

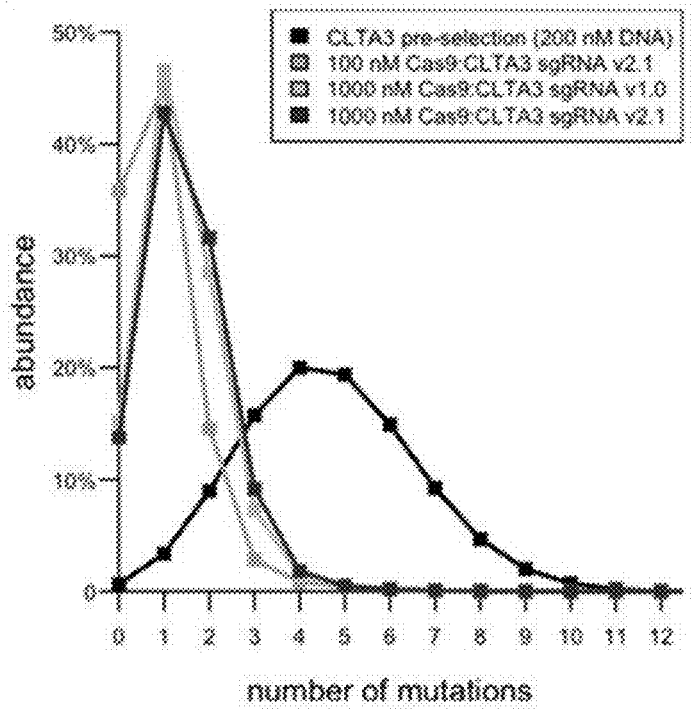

CLTA3 pre-selection (200 nM DNA)
100 nM Cas9:CLTA3 sgRNA v2.1
1000 nM Cas9:CLTA3 sgRNA v1.0
1000 nM Cas9:CLTA3 sgRNA v2.1
50%
40%
30%
20%
10%
0
abundance
0 1 2 3 4 5 6 7 8 9 10 11 12
number of mutations

FIG. 5D

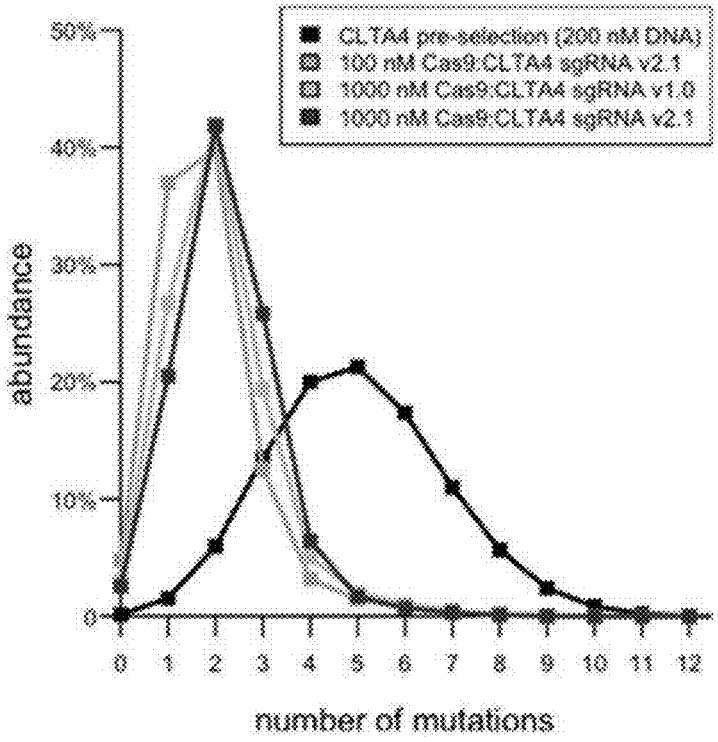

CLTA4 pre-selection (200 nM DNA)
100 nM Cas9:CLTA4 sgRNA v2.1
1000 nM Cas9:CLTA4 sgRNA v1.0
1000 nM Cas9:CLTA4 sgRNA v2.1
50%
40%
30%
20%
10%
0
abundance
0 1 2 3 4 5 6 7 8 9 10 11 12
number of mutations

FIG. 5E

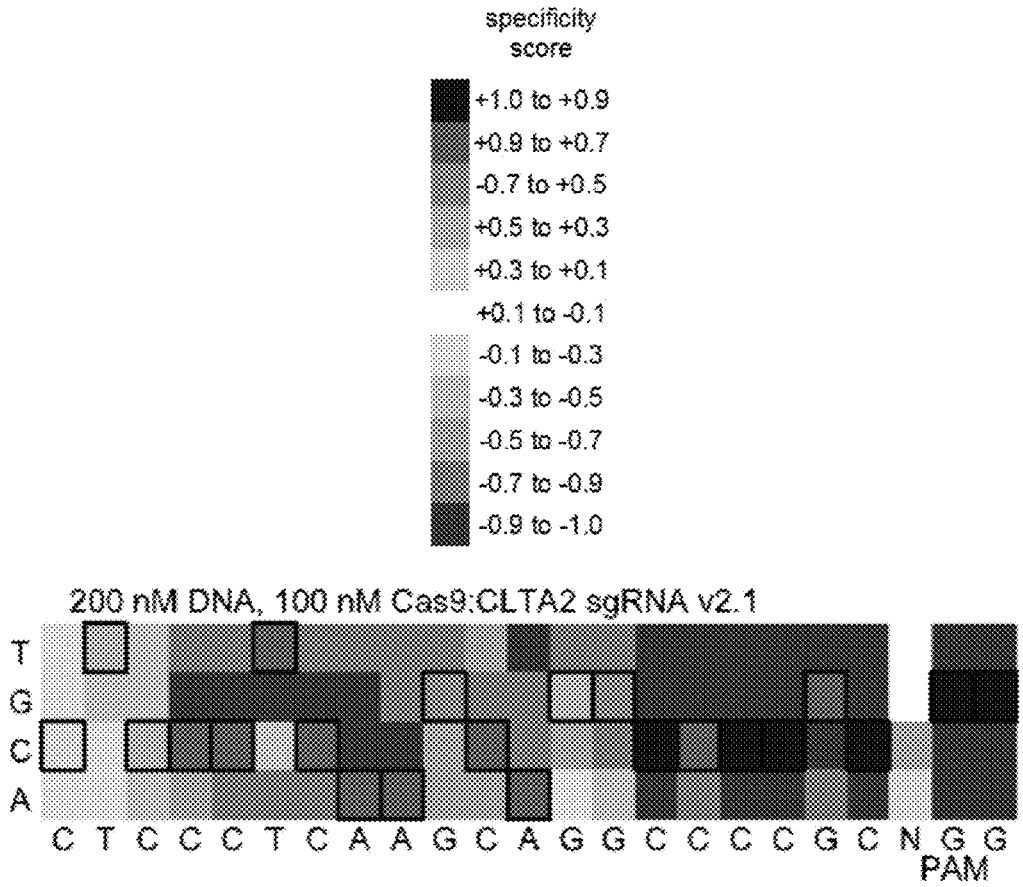

specificity score
+1.0 to +0.9
+0.9 to +0.7
-0.7 to +0.5
+0.5 to +0.3
+0.3 to +0.1
+0.1 to -0.1
-0.1 to -0.3
-0.3 to -0.5
-0.5 to -0.7
-0.7 to -0.9
-0.9 to -1.0
200 nM DNA, 100 nM Cas9:CLTA2 sgRNA v2.1
T
G
C
A
C T C C C T C A A G C A G G C C C C G C N G G
PAM

FIG. 6A

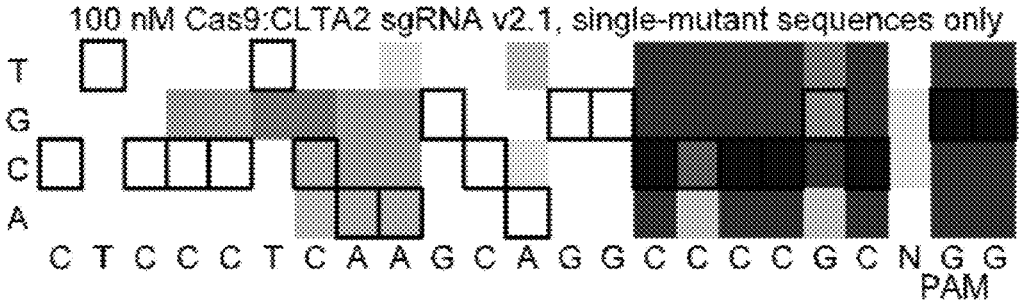

100 nM Cas9:CLTA2 sgRNA v2.1, single-mutant sequences only
T
G
C
A
C T C C C T C A A G C A G G C C C C G C N G G
PAM

FIG. 6B

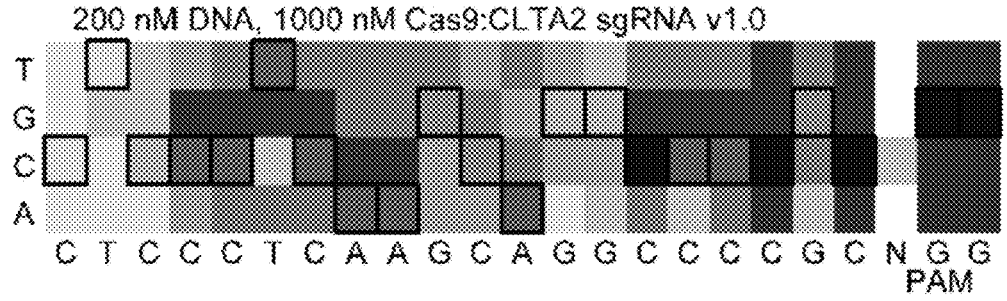


FIG. 6C

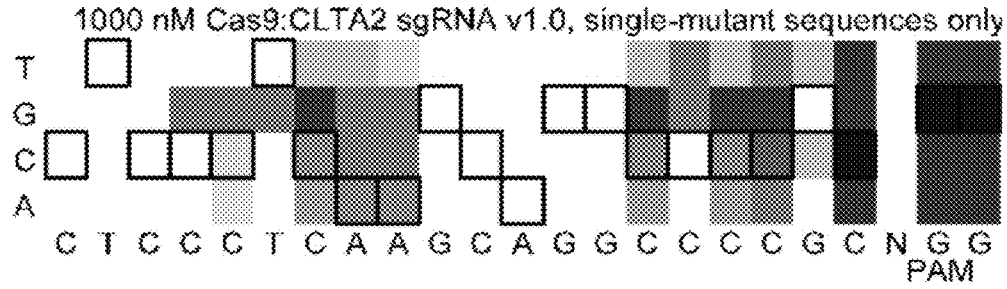


FIG. 6D

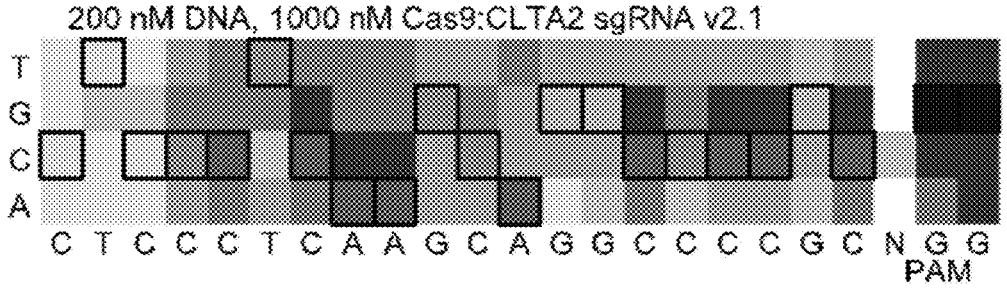


FIG. 6E

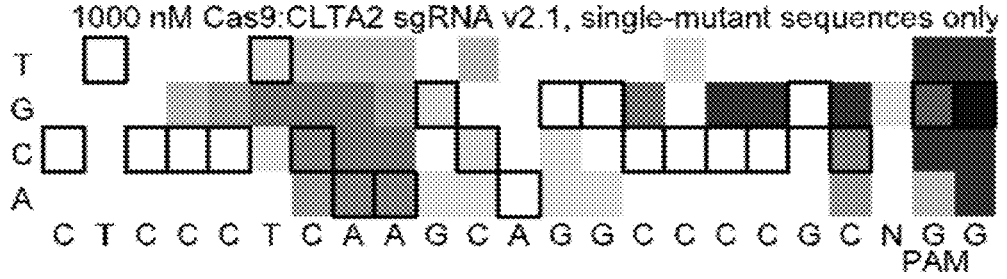


FIG. 6F

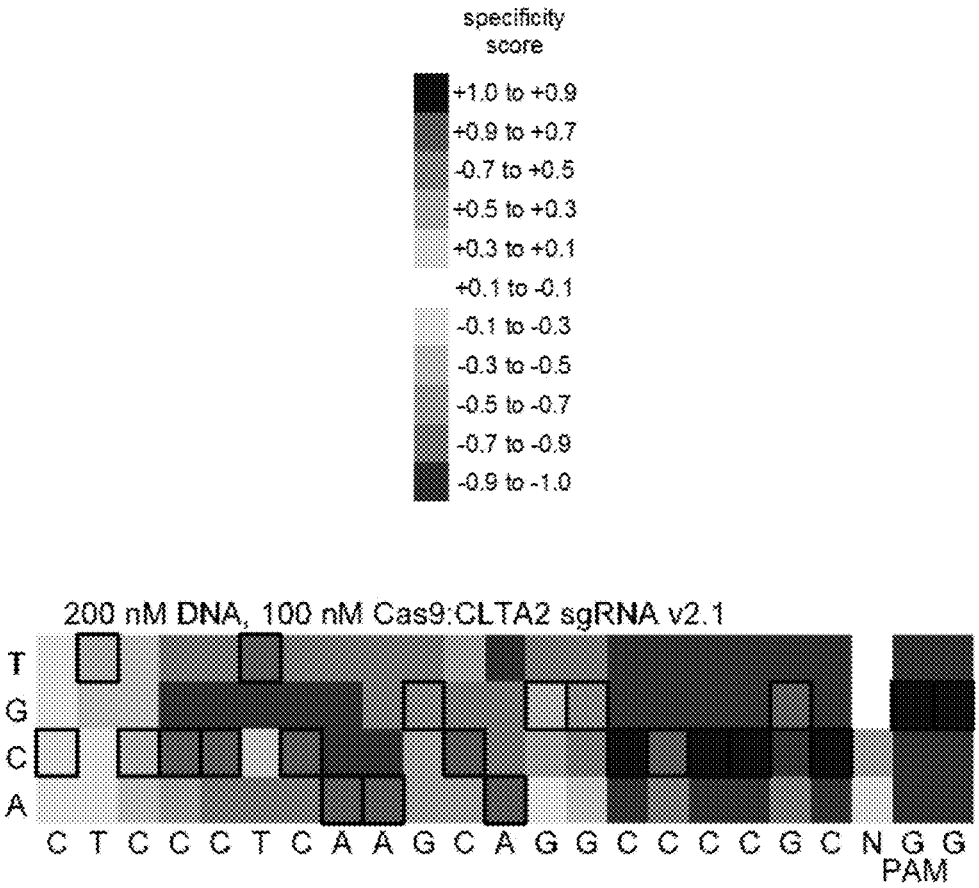

specificity score
+1.0 to +0.9
+0.9 to +0.7
-0.7 to +0.5
+0.5 to +0.3
+0.3 to +0.1
+0.1 to -0.1
-0.1 to -0.3
-0.3 to -0.5
-0.5 to -0.7
-0.7 to -0.9
-0.9 to -1.0
200 nM DNA, 100 nM Cas9:CLTA2 sgRNA v2.1
T
G
C
A
C T C C C T C A A G C A G G C C C C G C N G G
PAM

FIG. 7A

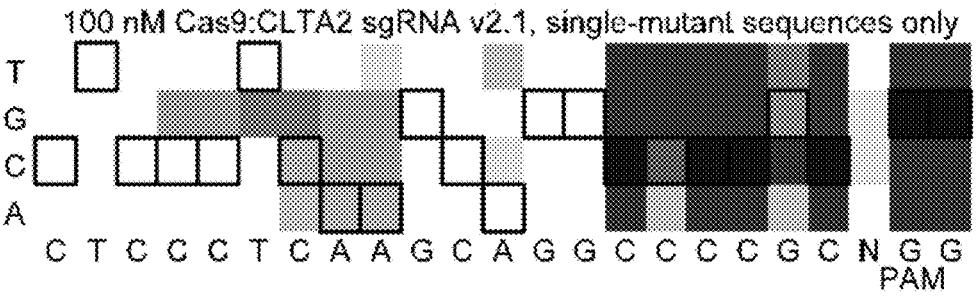

100 nM Cas9:CLTA2 sgRNA v2.1, single-mutant sequences only
T
G
C
A
C T C C C T C A A G C A G G C C C C G C N G G
PAM

FIG. 7B

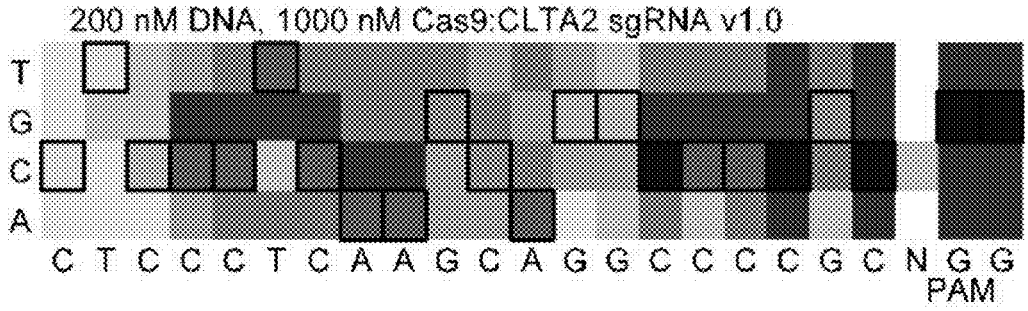


FIG. 7C

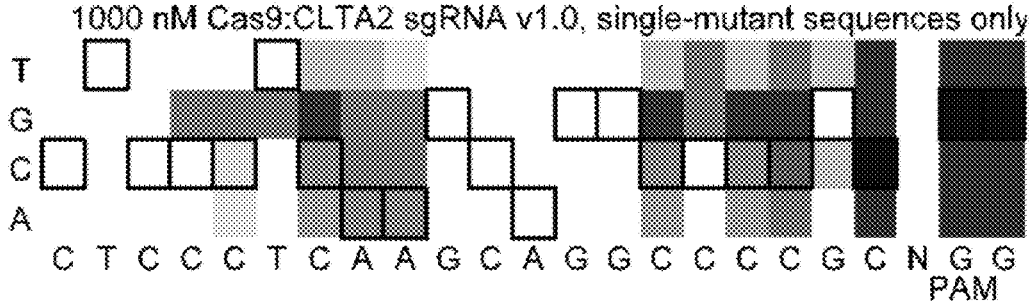


FIG. 7D

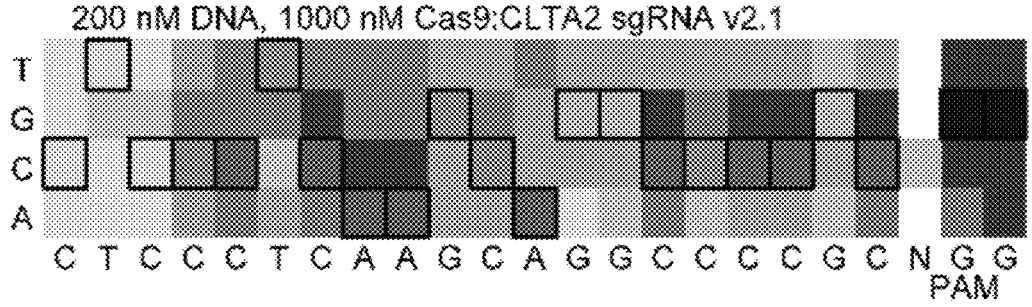


FIG. 7E

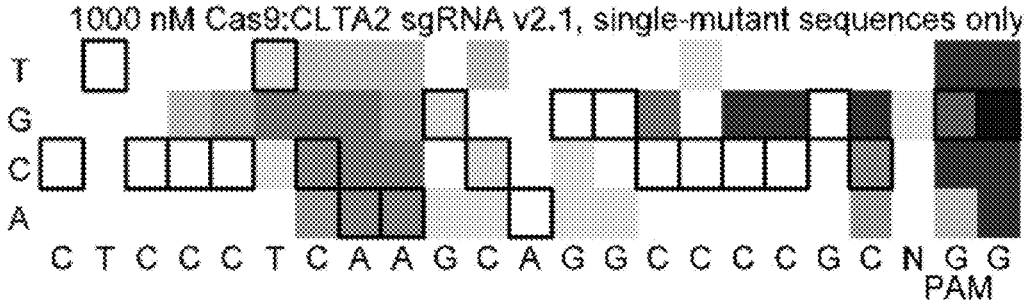


FIG. 7F

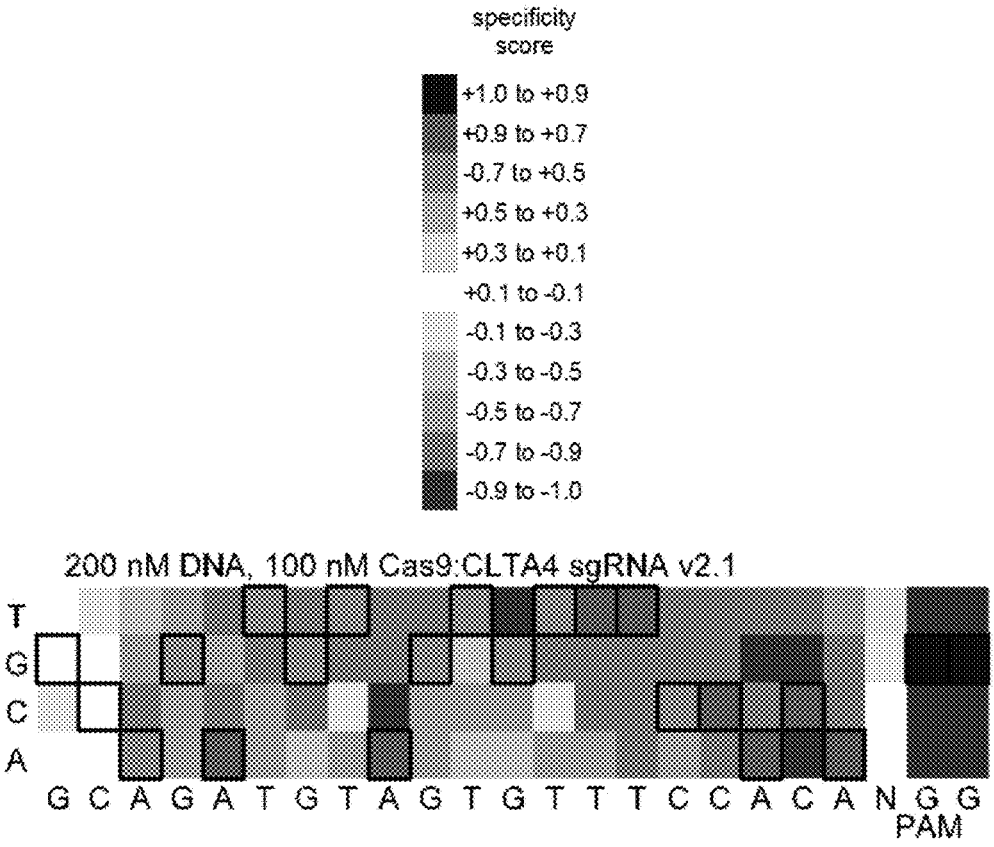

specificity score
+1.0 to +0.9
+0.9 to +0.7
-0.7 to +0.5
+0.5 to +0.3
+0.3 to +0.1
+0.1 to -0.1
-0.1 to -0.3
-0.3 to -0.5
-0.5 to -0.7
-0.7 to -0.9
-0.9 to -1.0
200 nM DNA, 100 nM Cas9:CLTA4 sgRNA v2.1
T
G
C
A
G C A G A T G T A G T G T T T C C A C A N G G
PAM

FIG. 8A

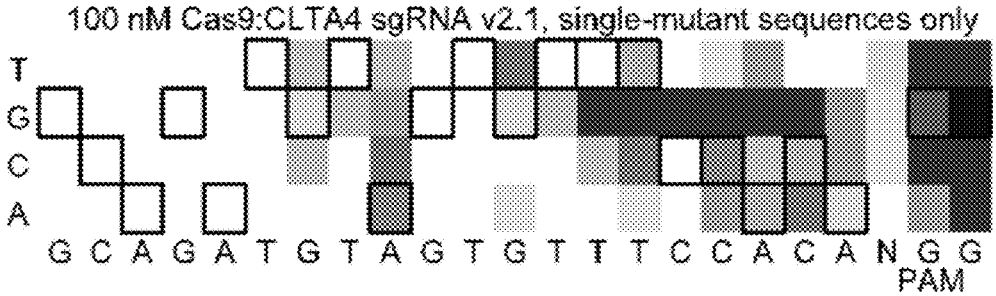

100 nM Cas9:CLTA4 sgRNA v2.1, single-mutant sequences only
T
G
C
A
G C A G A T G T A G T G T T T C C A C A N G G
PAM

FIG. 8B

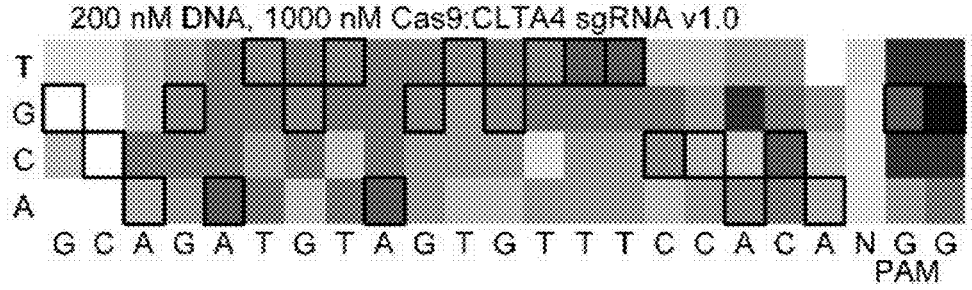


FIG. 8C

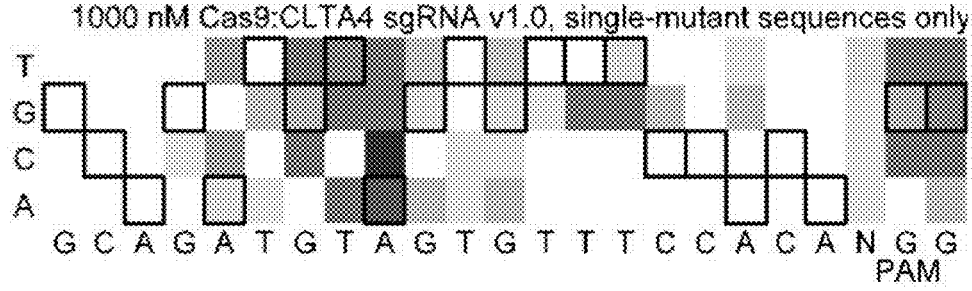


FIG. 8D

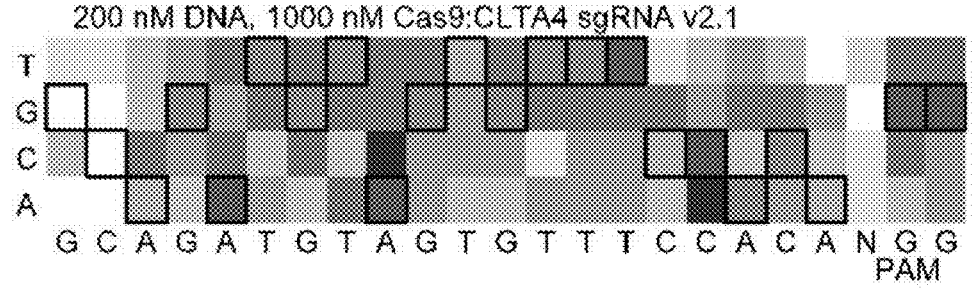


FIG. 8E

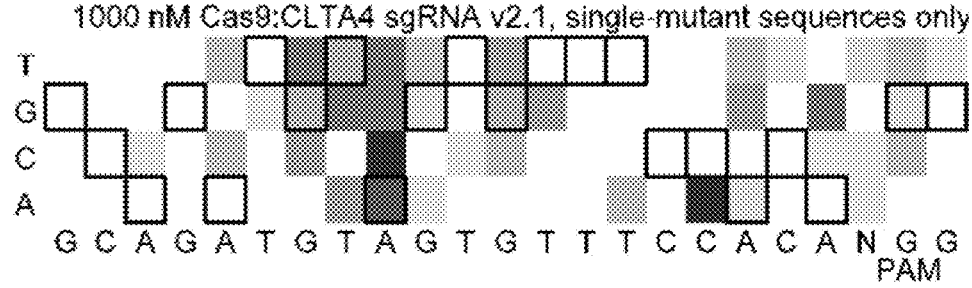


FIG. 8F

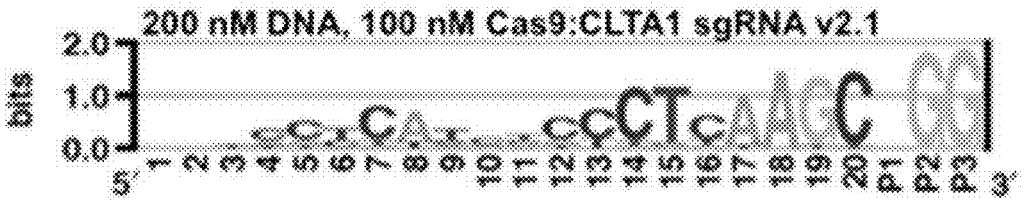


FIG. 9A

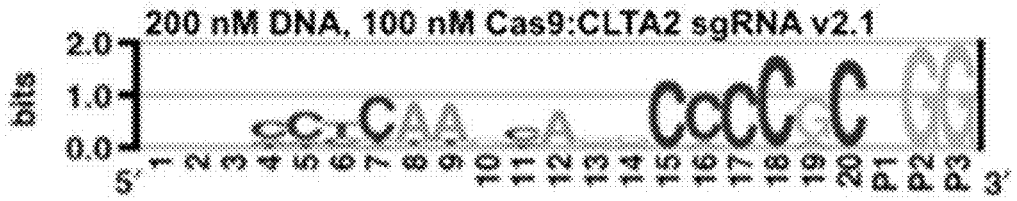


FIG. 9B

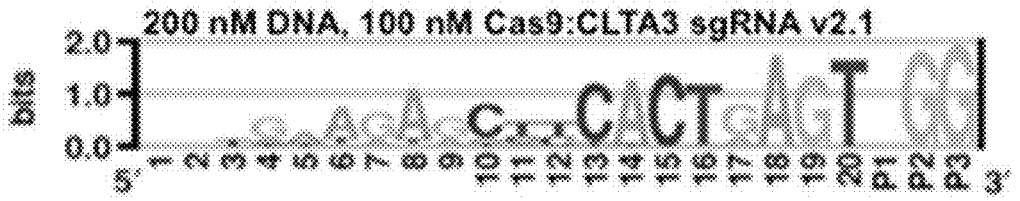


FIG. 9C

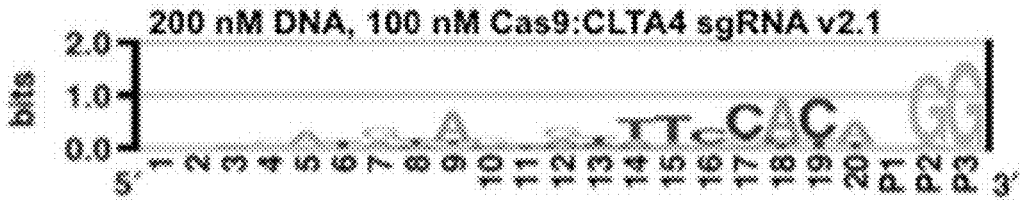


FIG. 9D

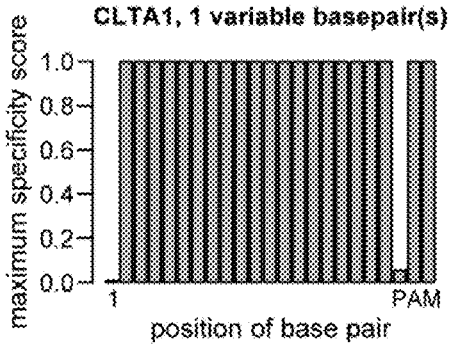


FIG. 10A

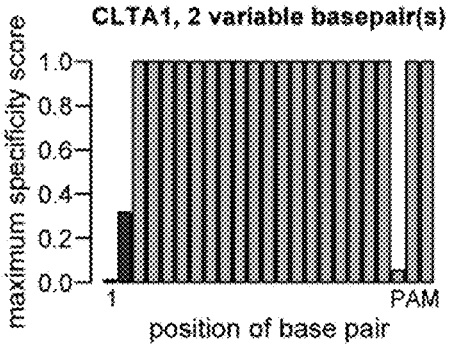


FIG. 10B

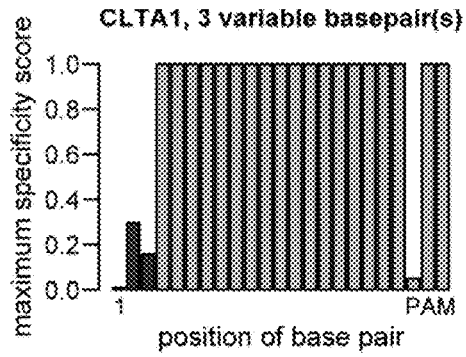


FIG. 10C

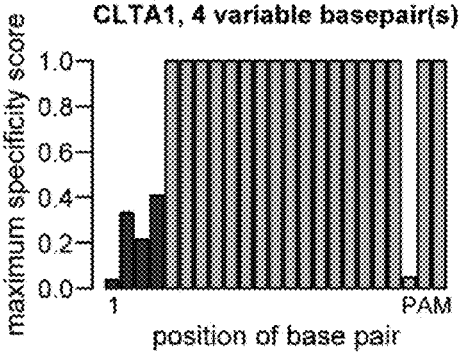


FIG. 10D

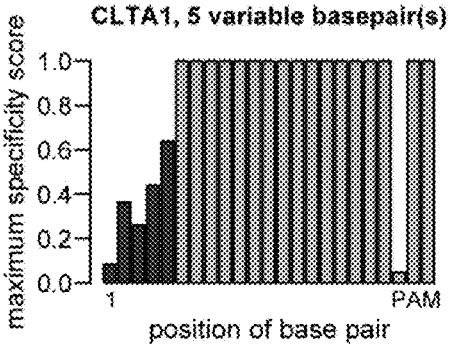


FIG. 10E

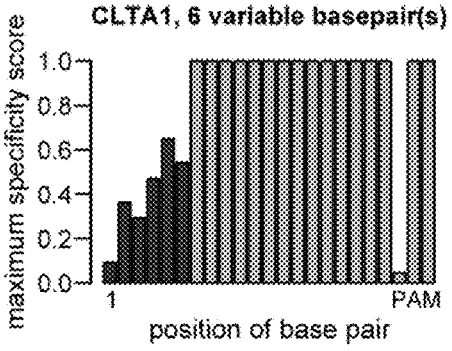


FIG. 10F

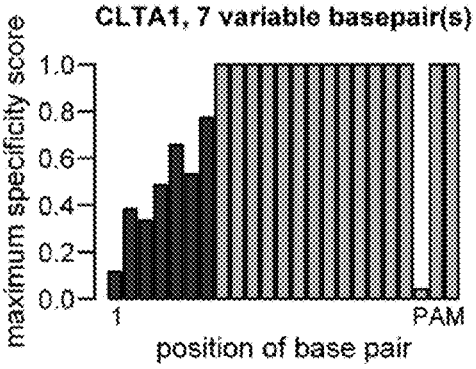


FIG. 10G

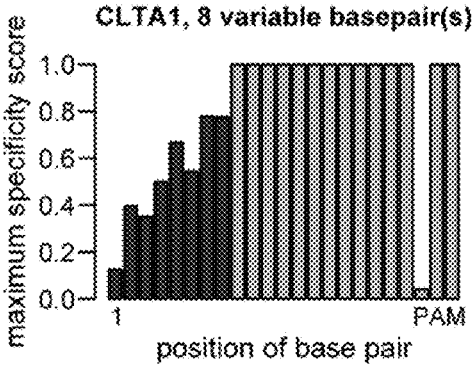


FIG. 10H

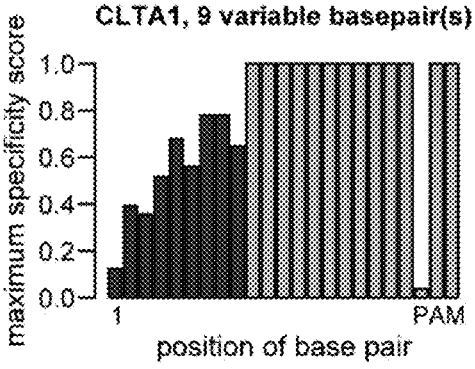


FIG. 10I

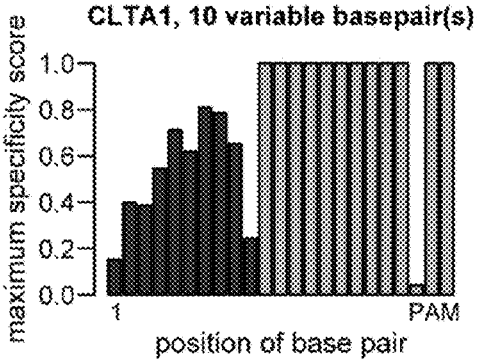


FIG. 10J

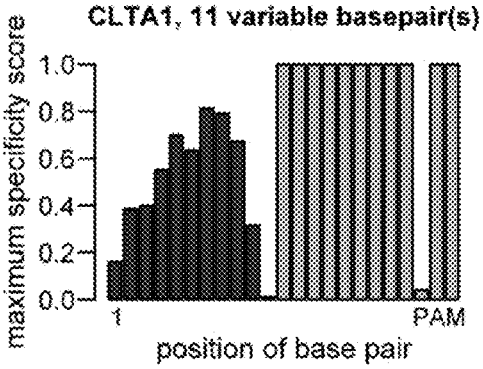


FIG. 10K

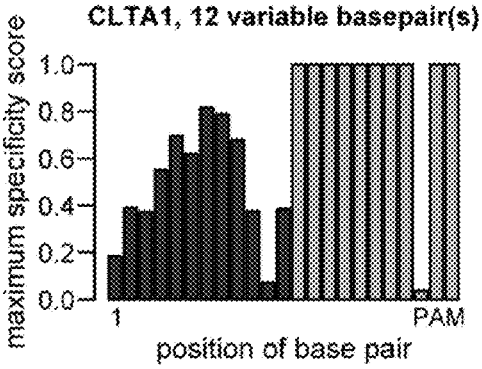


FIG. 10L

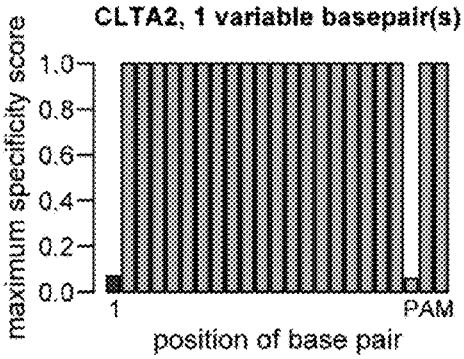


FIG. 11A

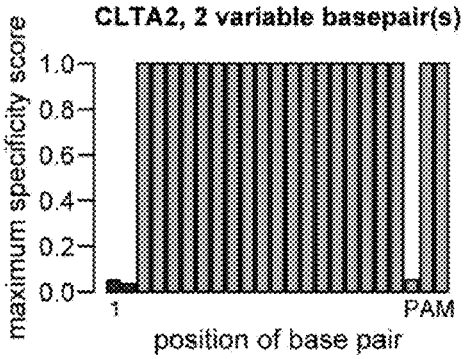


FIG. 11B

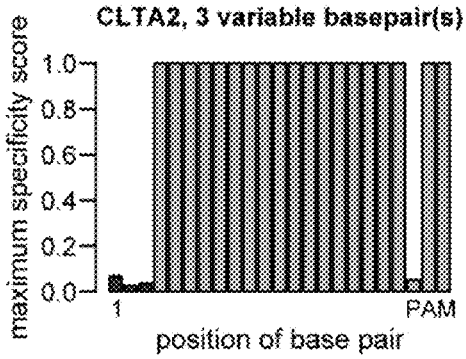


FIG. 11C

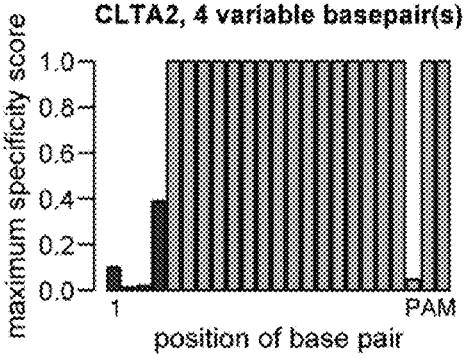


FIG. 11D

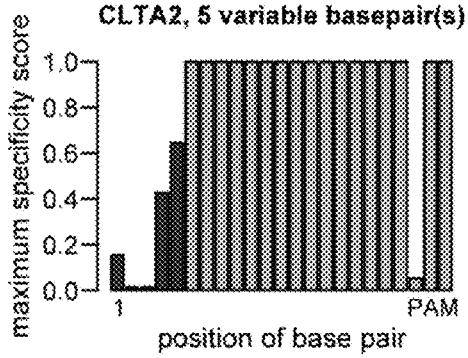


FIG. 11E

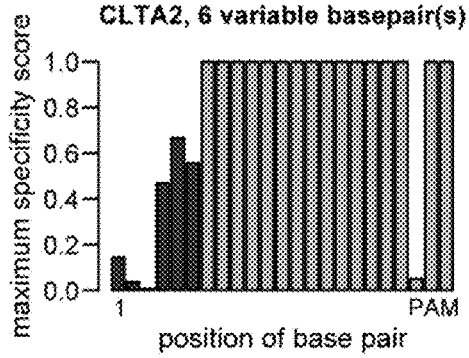


FIG. 11F

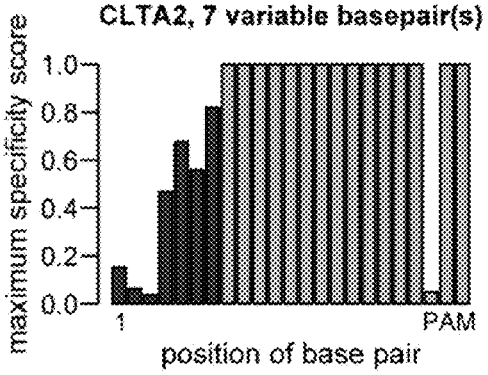


FIG. 11G

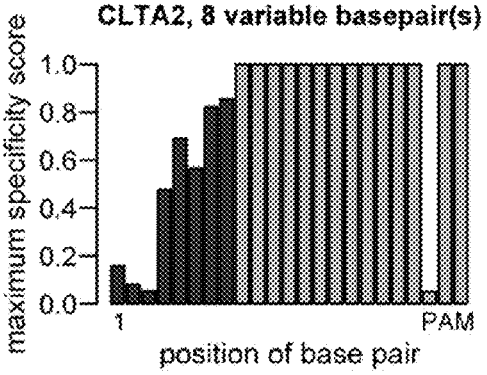


FIG. 11H

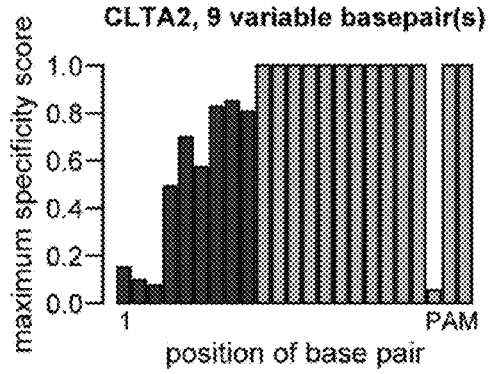


FIG. 11I

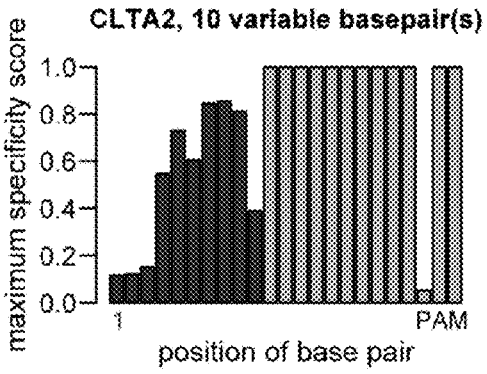


FIG. 11J

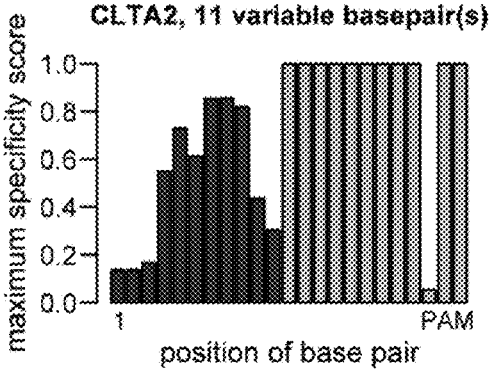


FIG. 11K

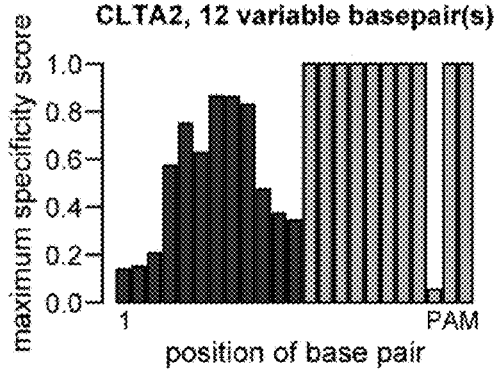


FIG. 11L

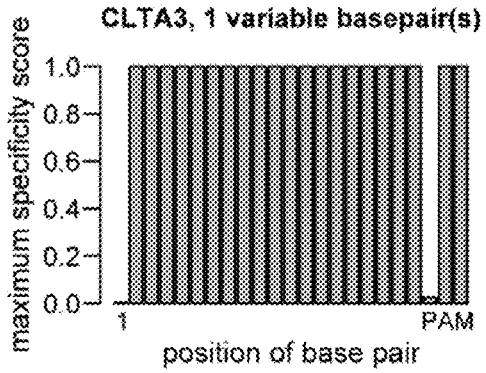


FIG. 12A

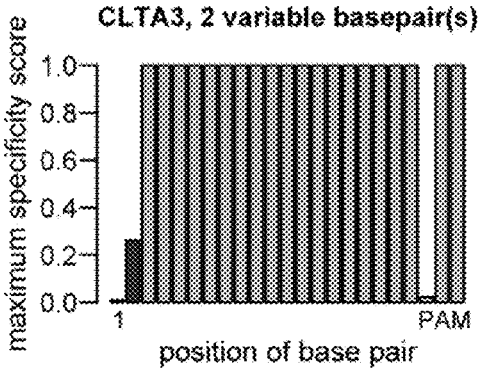


FIG. 12B

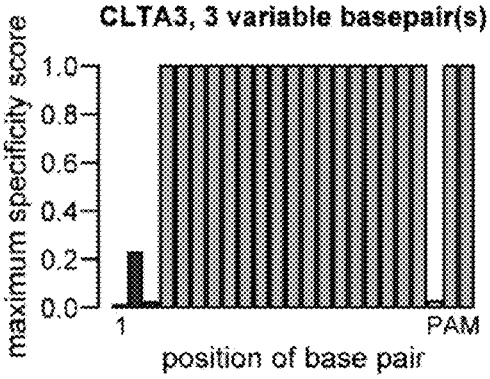


FIG. 12C

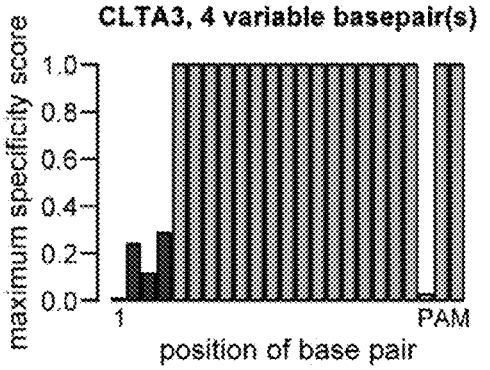


FIG. 12D

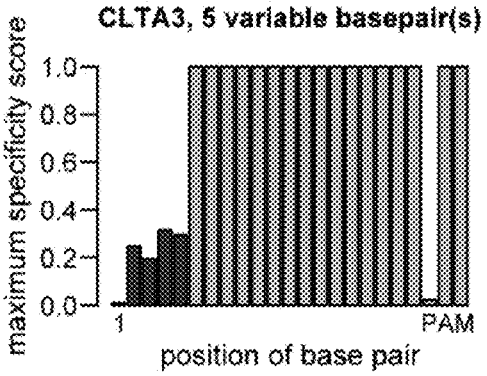


FIG. 12E

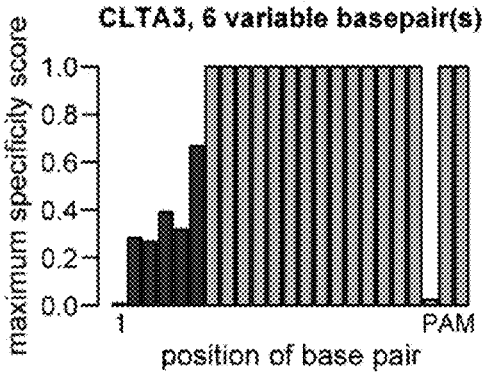


FIG. 12F

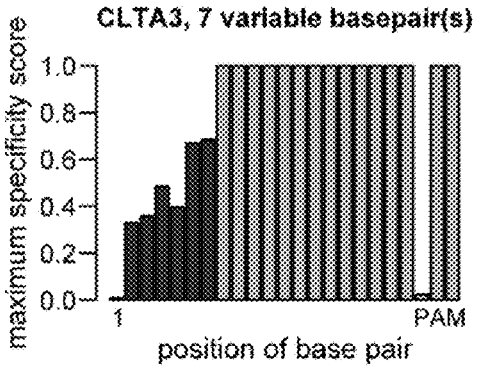


FIG. 12G

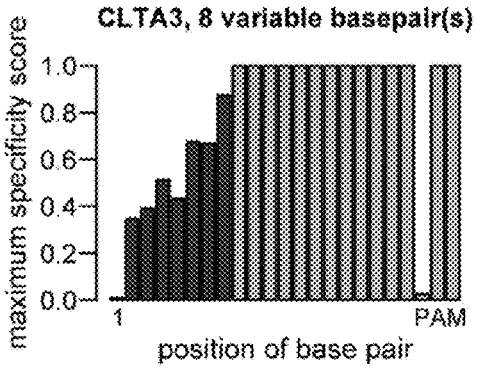


FIG. 12H

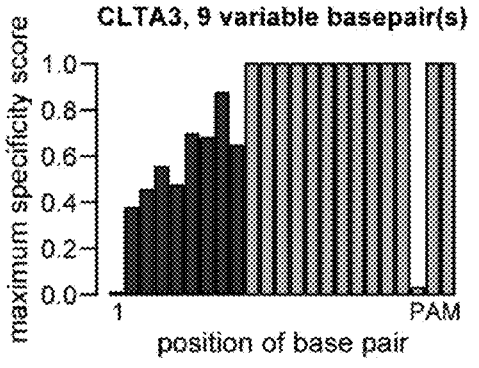


FIG. 12I

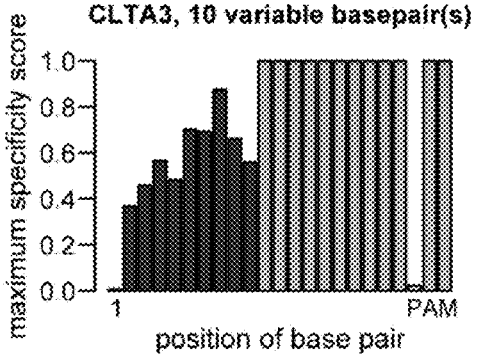


FIG. 12J

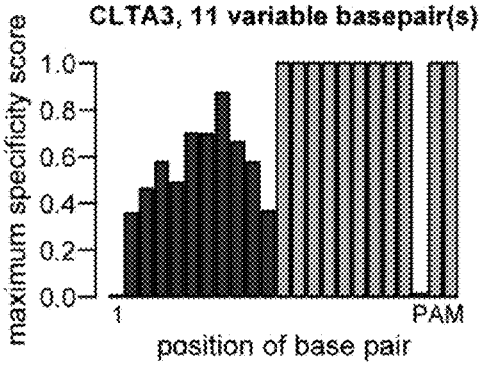


FIG. 12K

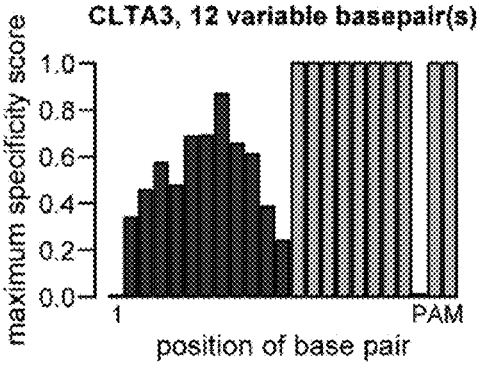


FIG. 12L

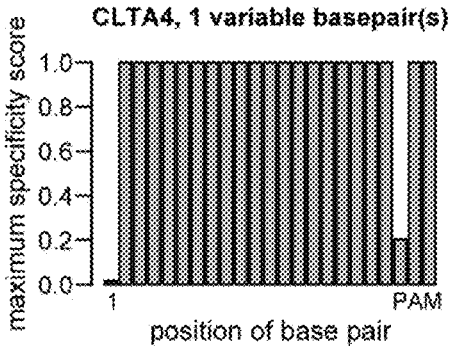


FIG. 13A

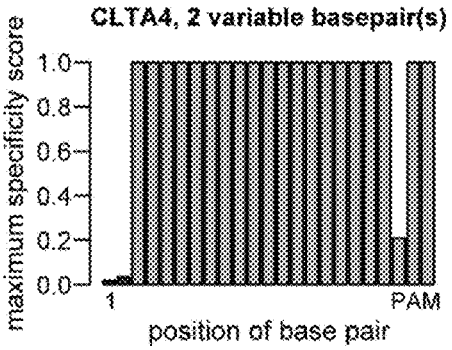


FIG. 13B

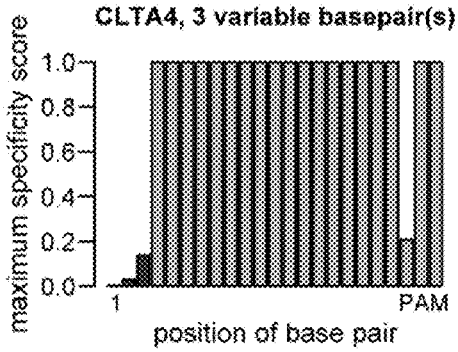


FIG. 13C

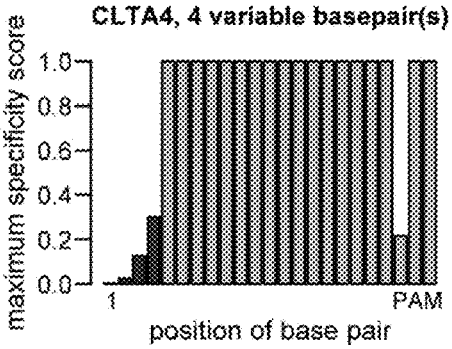


FIG. 13D

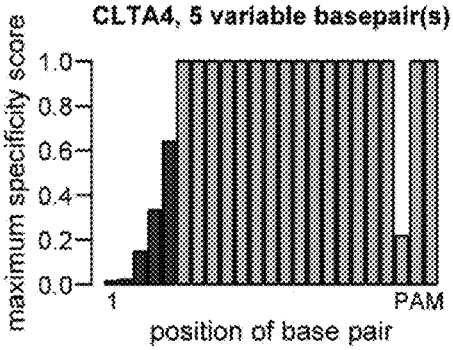


FIG. 13E

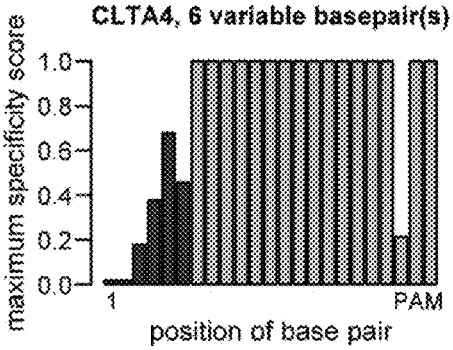


FIG. 13F

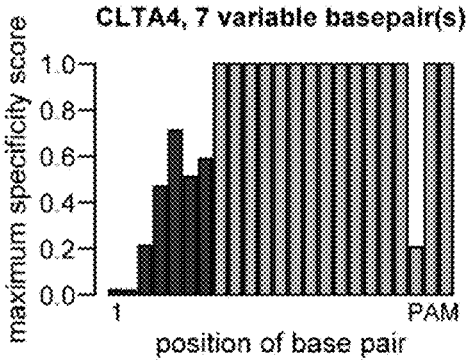


FIG. 13G

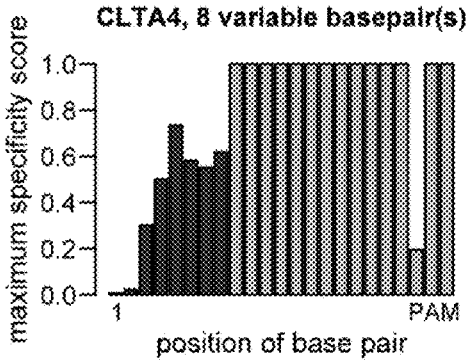


FIG. 13H

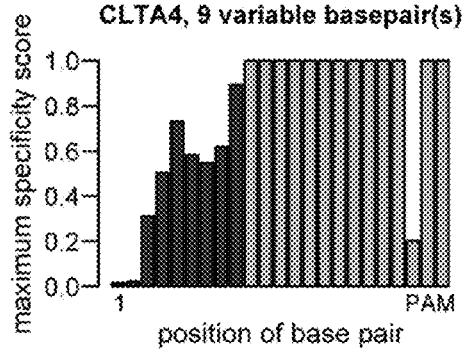


FIG. 13I

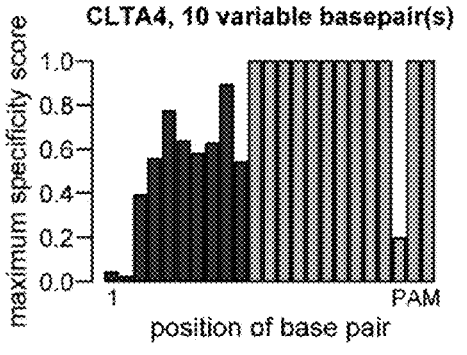


FIG. 13J

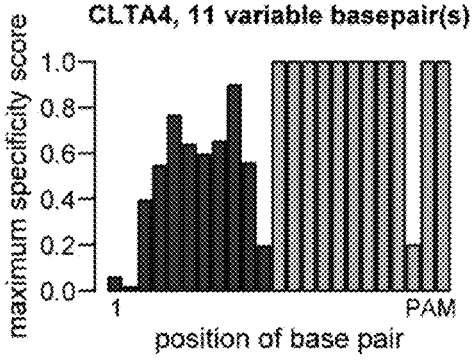


FIG. 13K

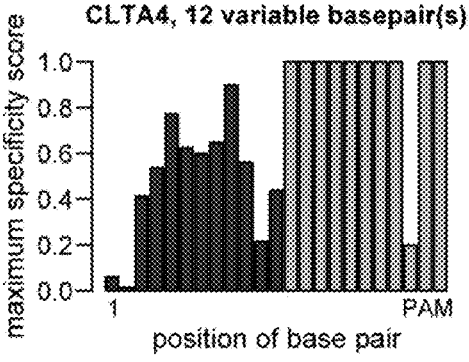


FIG. 13L

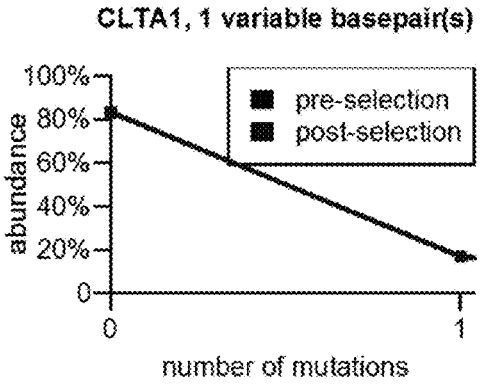


FIG. 14A

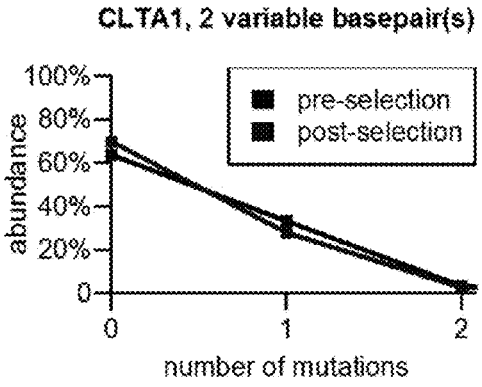


FIG. 14B

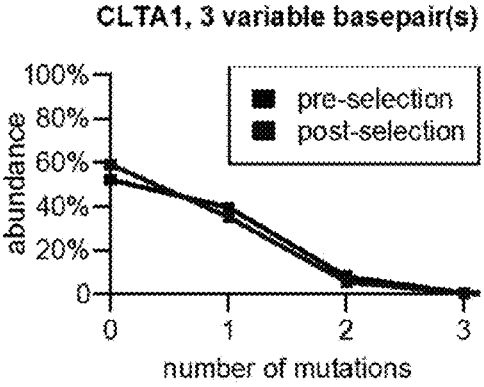


FIG. 14C

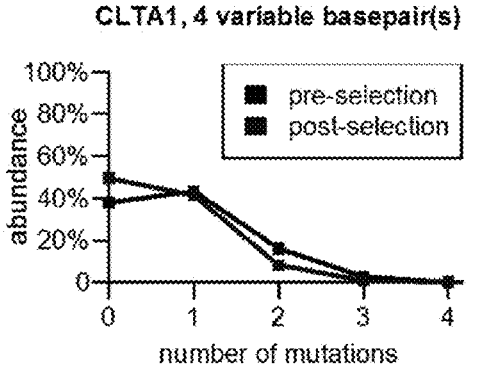


FIG. 14D

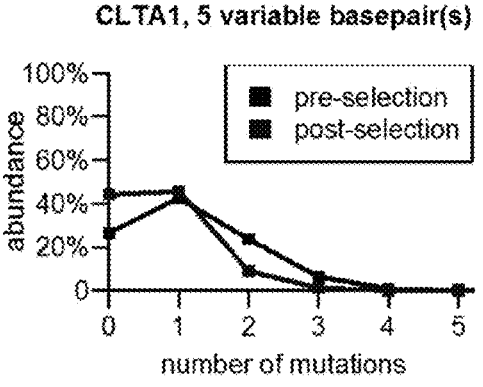


FIG. 14E

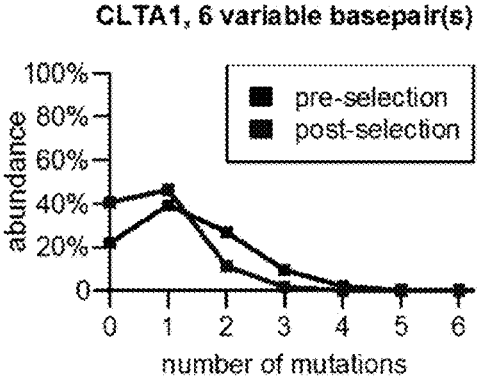


FIG. 14F

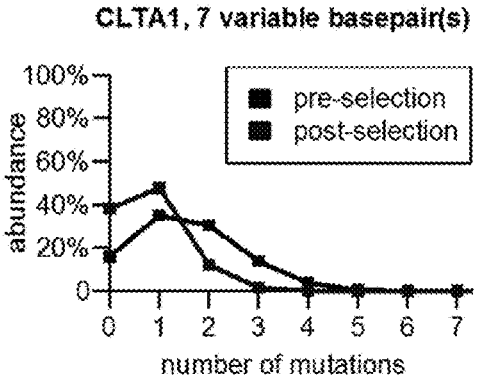


FIG. 14G

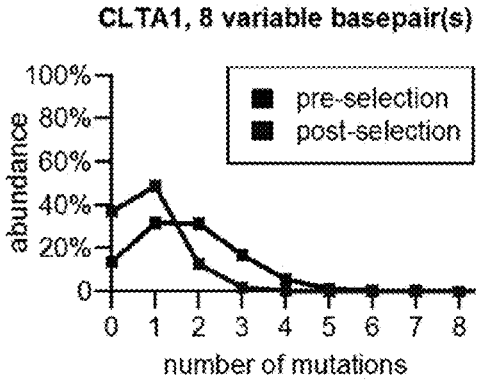


FIG. 14H

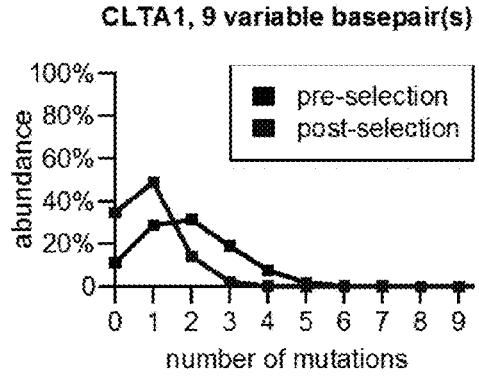


FIG. 14I

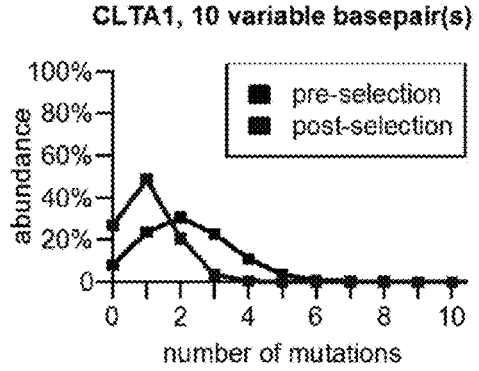


FIG. 14J

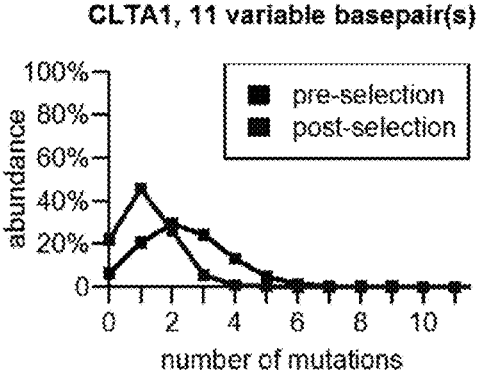


FIG. 14K

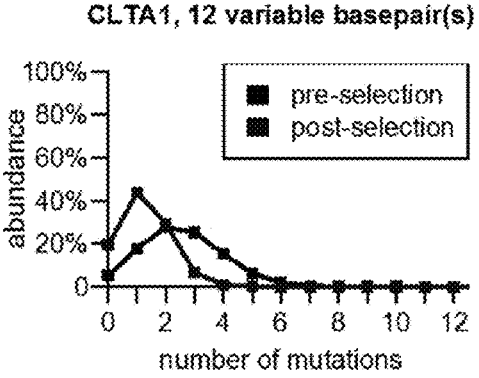


FIG. 14L

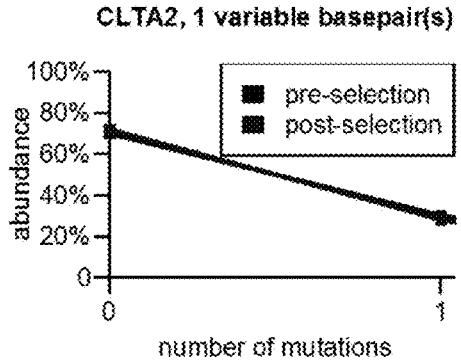


FIG. 15A

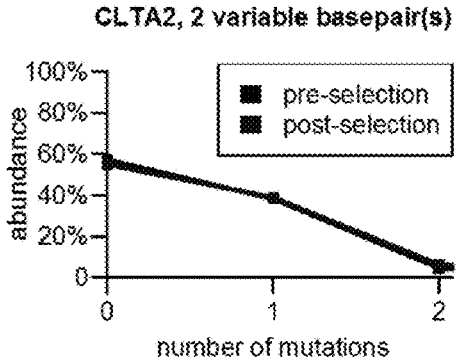


FIG. 15B

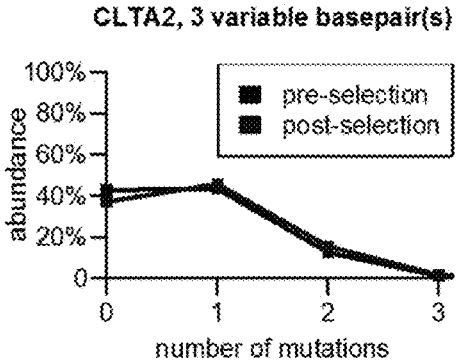


FIG. 15C

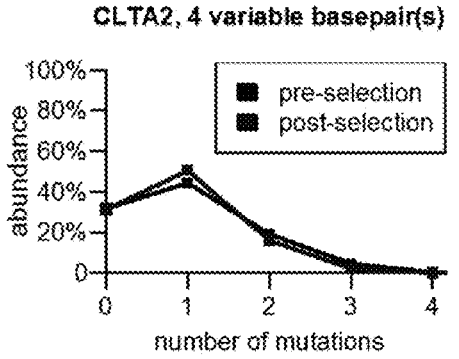


FIG. 15D

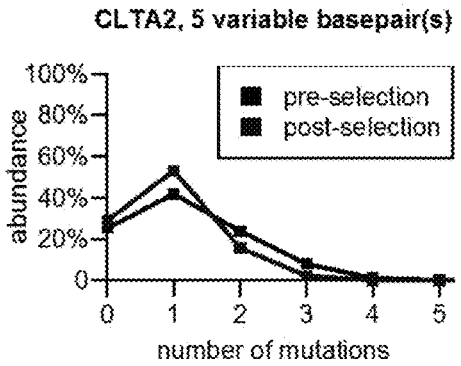


FIG. 15E

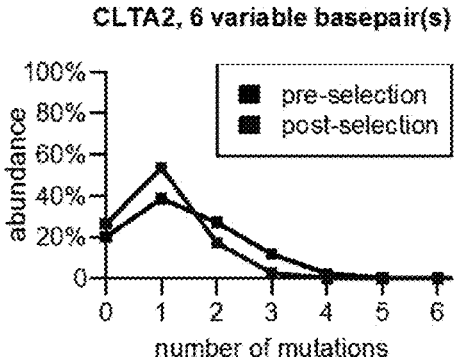


FIG. 15F

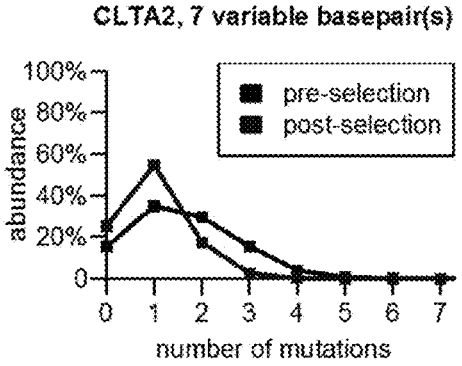


FIG. 15G

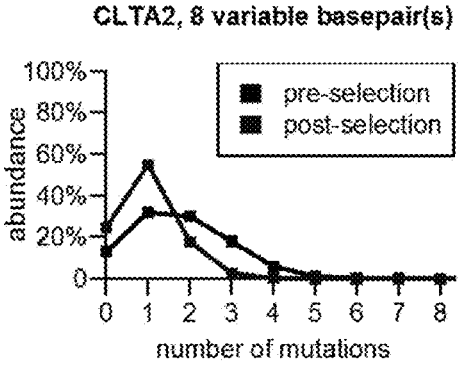


FIG. 15H

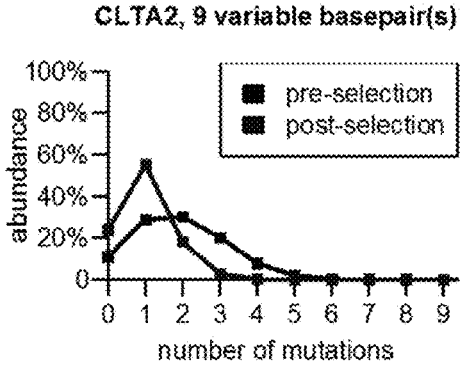


FIG. 15I

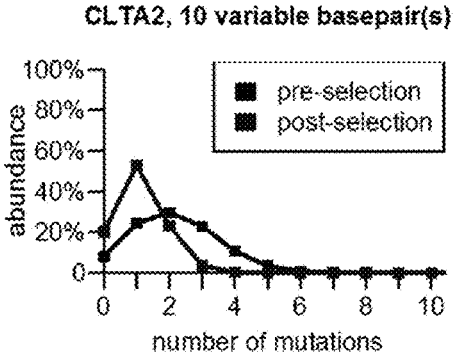


FIG. 15J

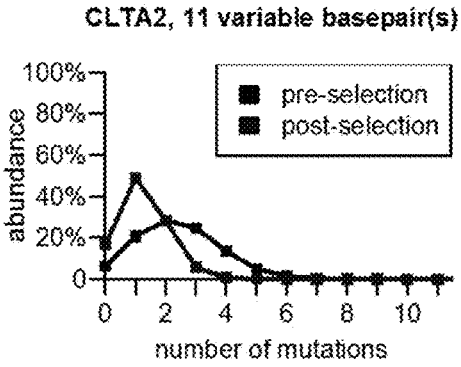


FIG. 15K

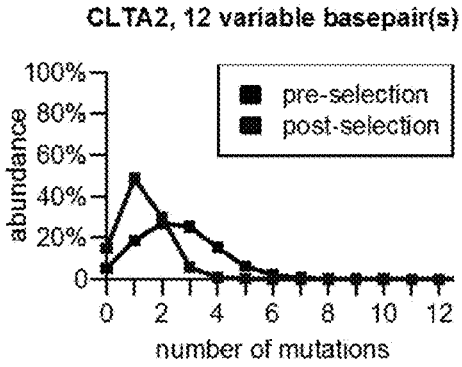


FIG. 15L

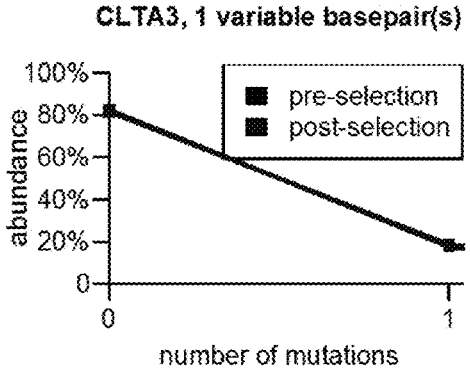


FIG. 16A

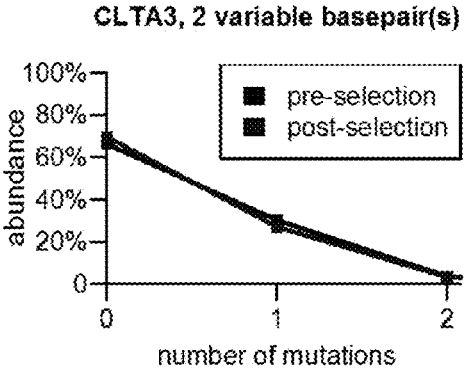


FIG. 16B

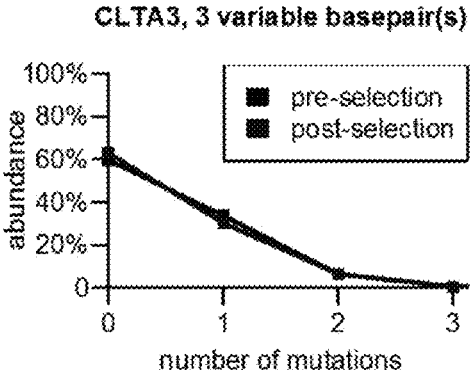


FIG. 16C

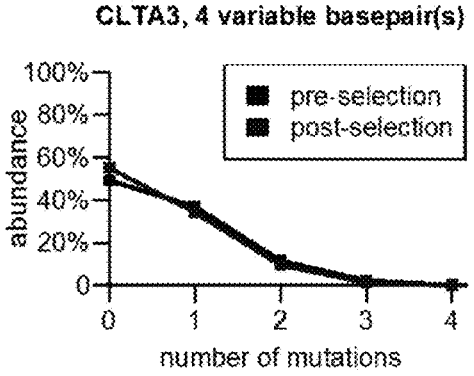


FIG. 16D

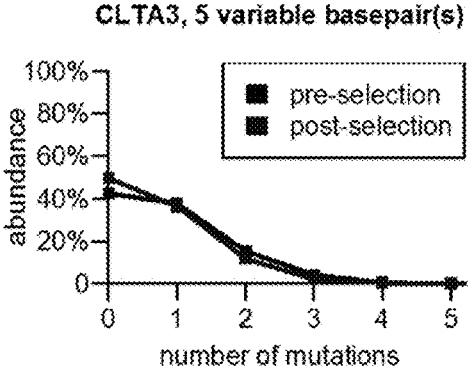


FIG. 16E

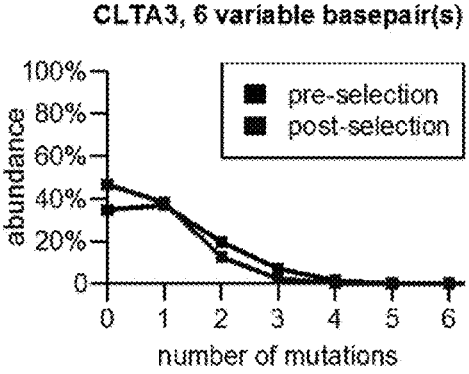


FIG. 16F

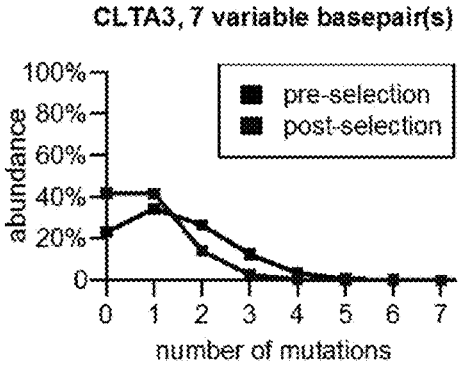


FIG. 16G

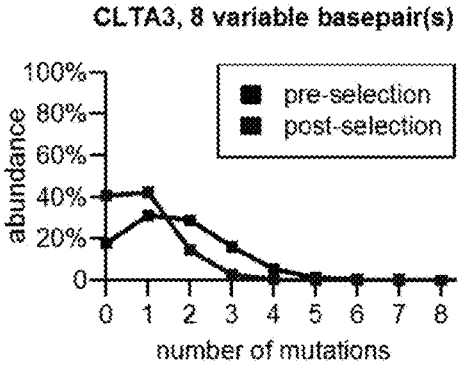


FIG. 16H

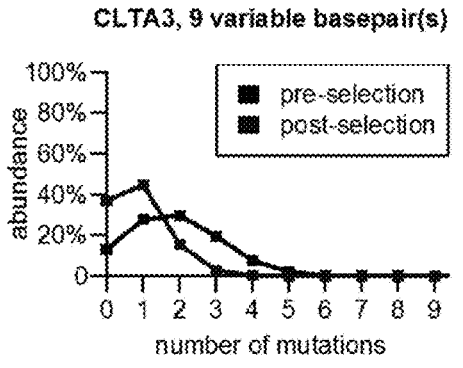


FIG. 16I

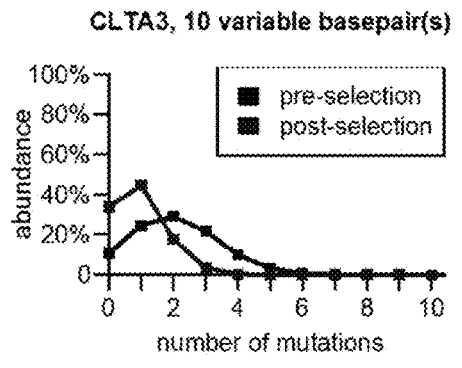


FIG. 16J

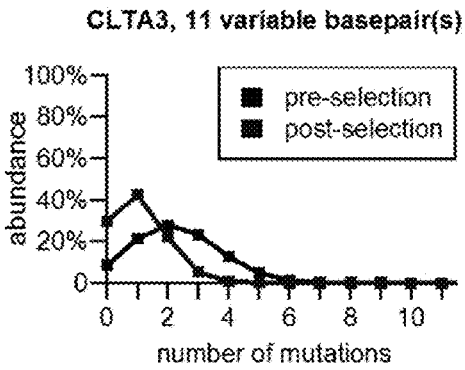


FIG. 16K

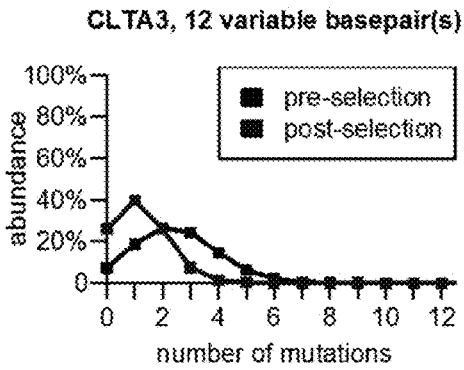


FIG. 16L

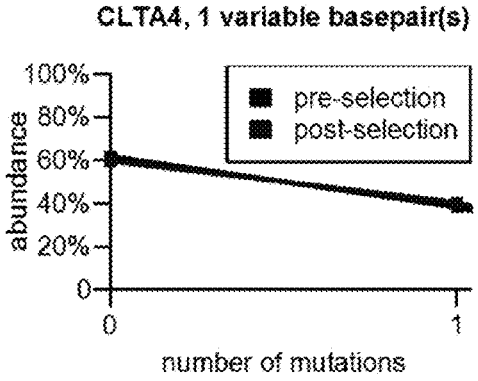


FIG. 17A

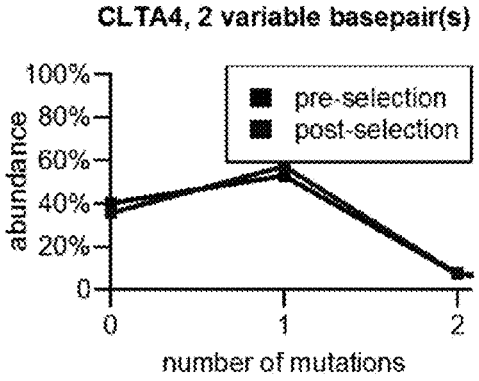


FIG. 17B

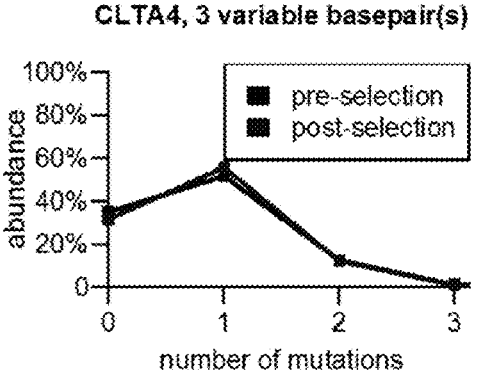


FIG. 17C

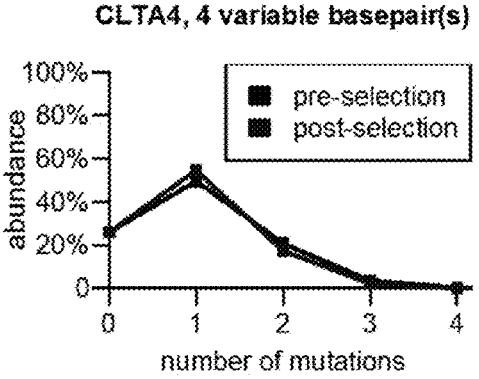


FIG. 17D

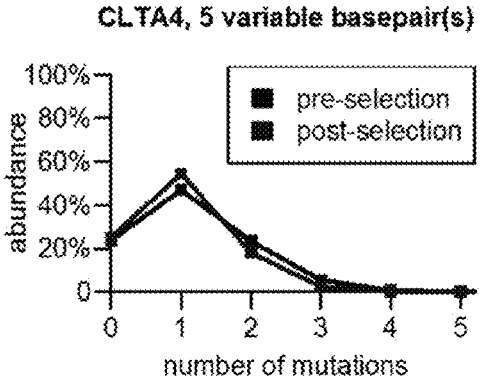


FIG. 17E

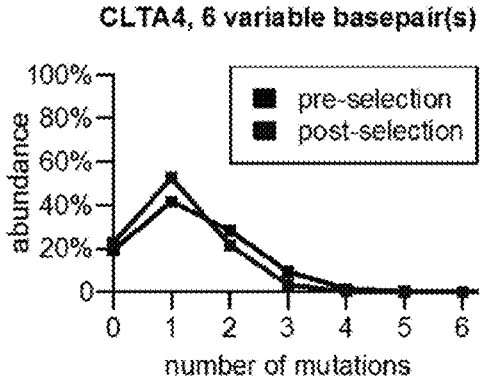


FIG. 17F

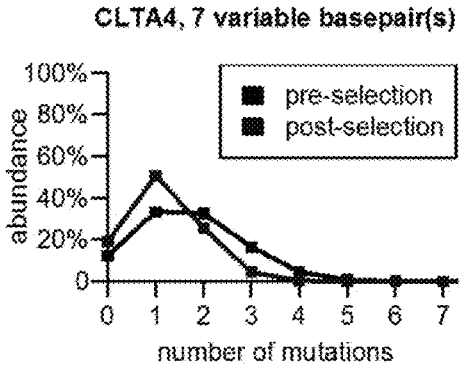


FIG. 17G

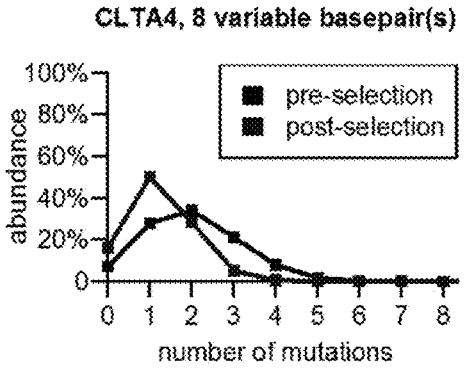


FIG. 17H

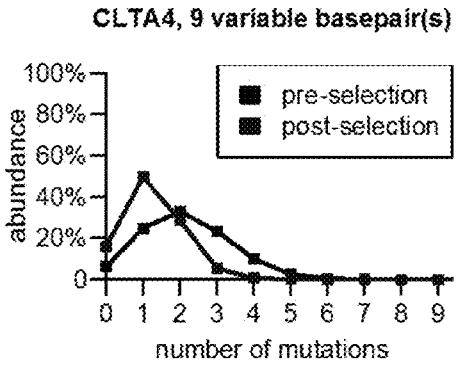


FIG. 17I

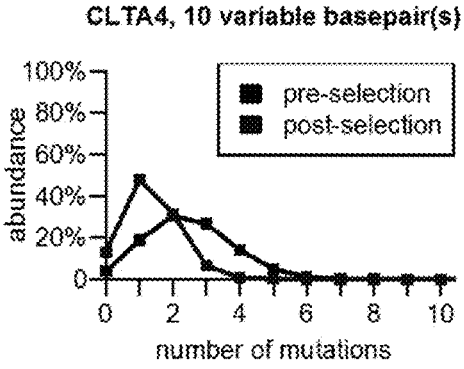


FIG. 17J

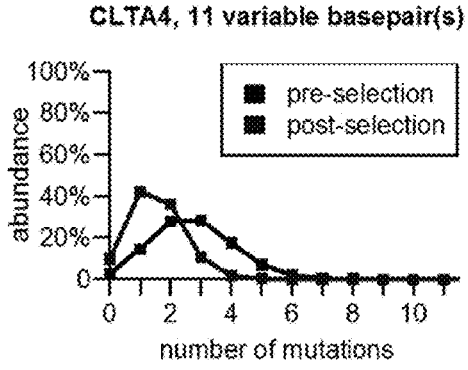


FIG. 17K

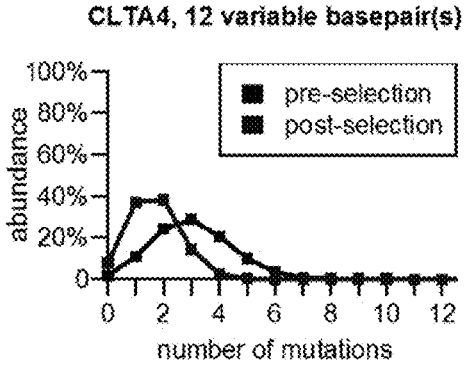


FIG. 17L

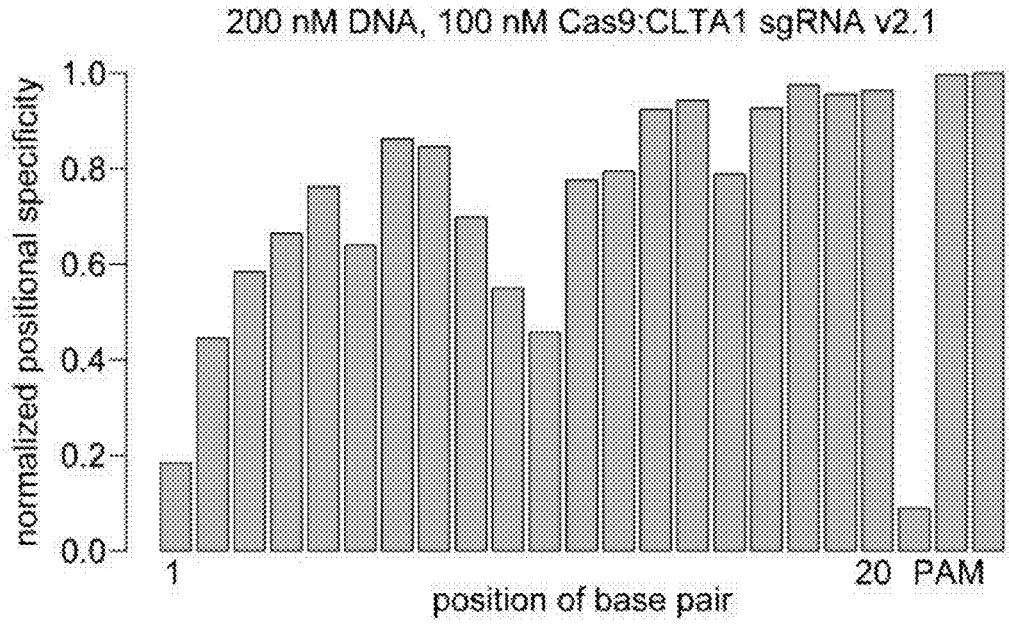


FIG. 18A

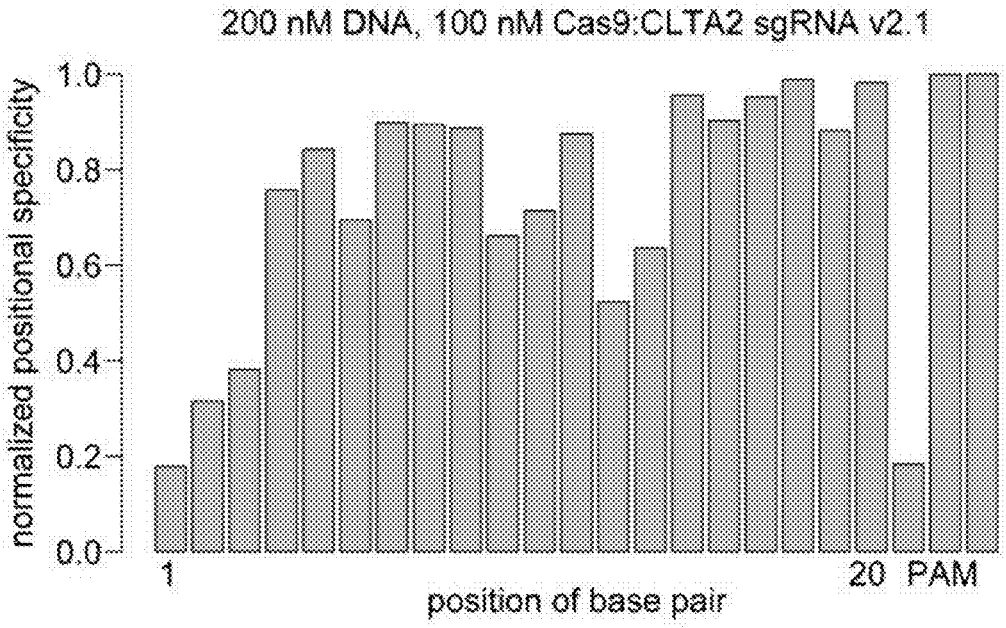


FIG. 18B

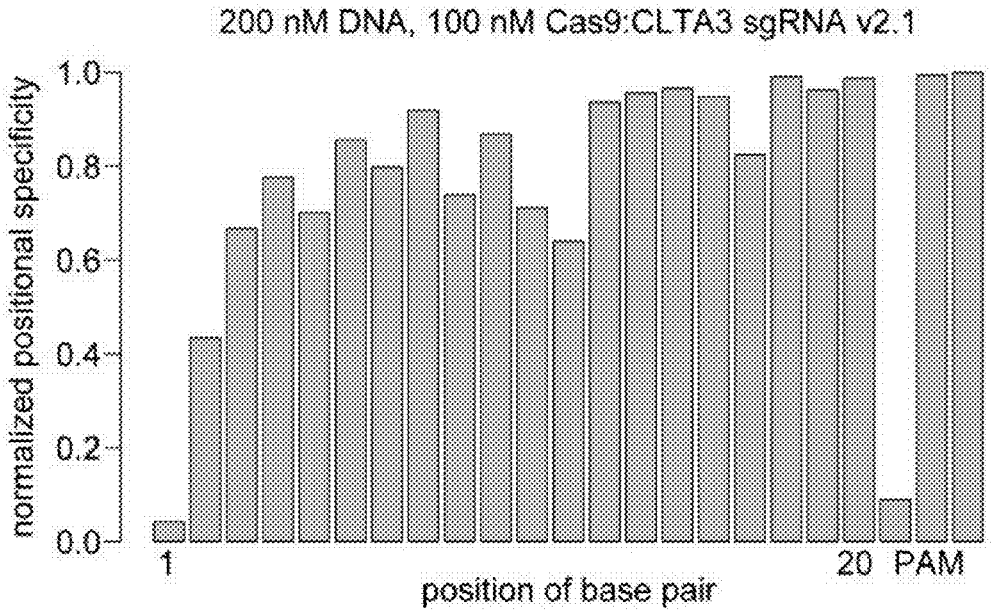


FIG. 18C

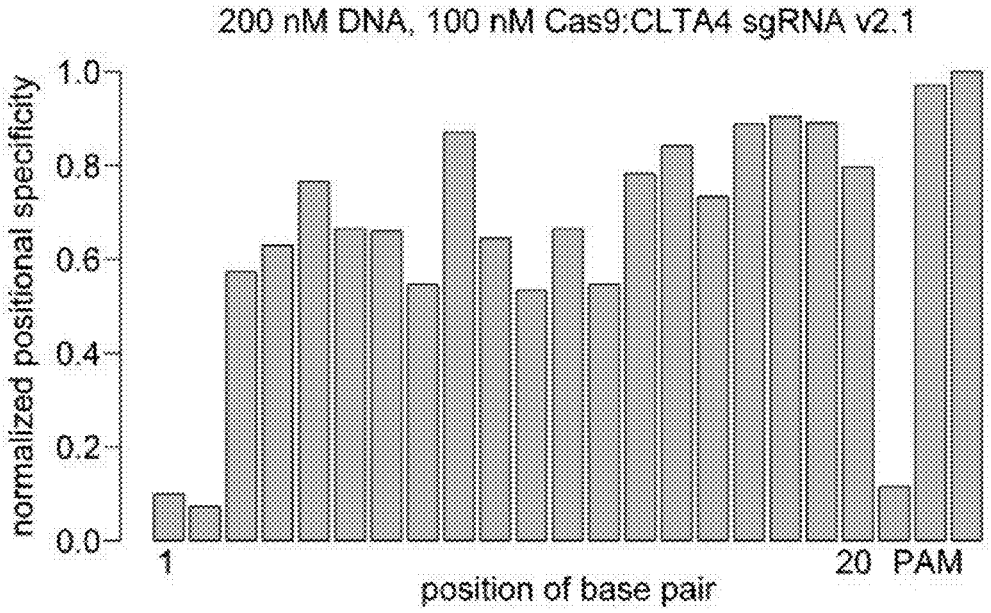


FIG. 18D

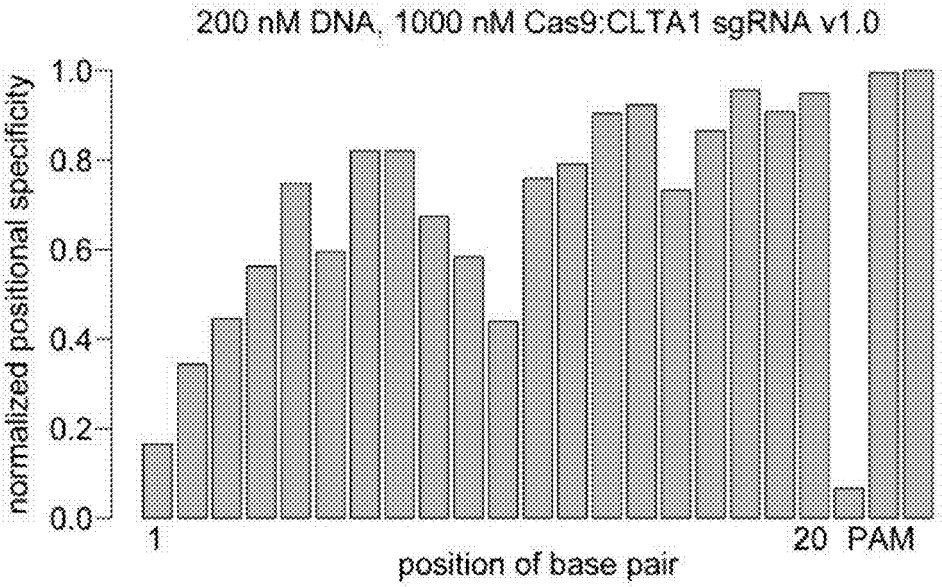


FIG. 19A

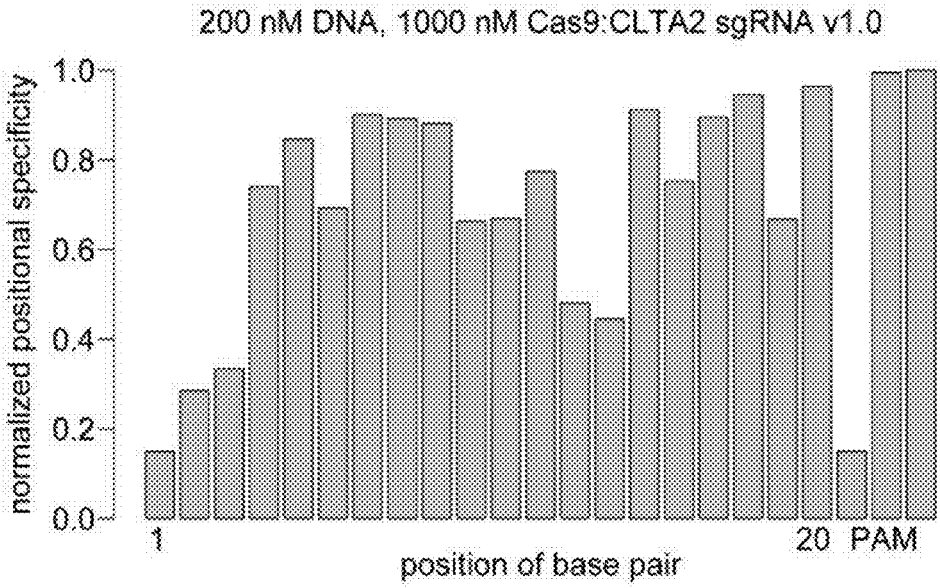


FIG. 19B

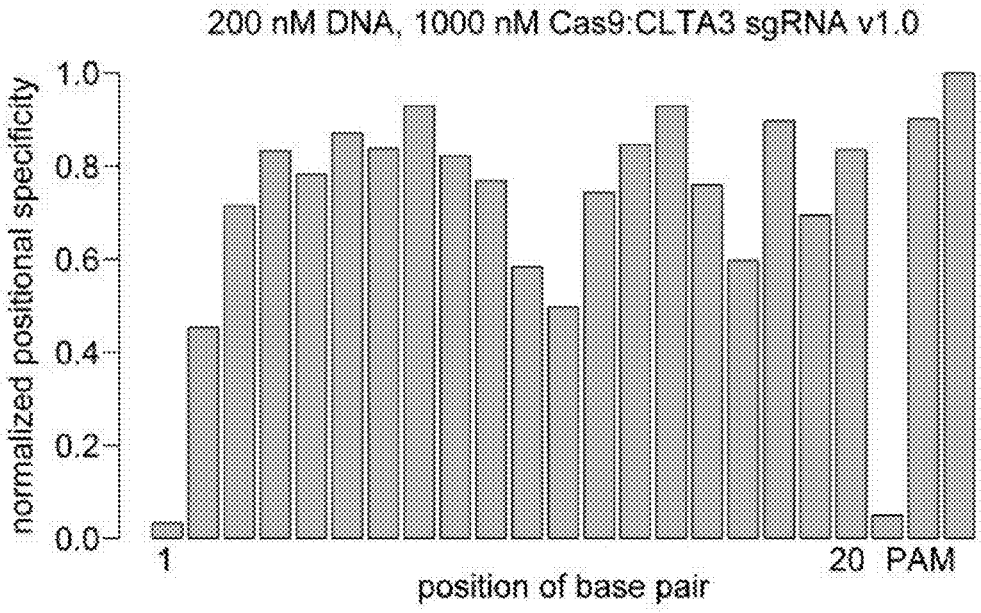


FIG. 19C

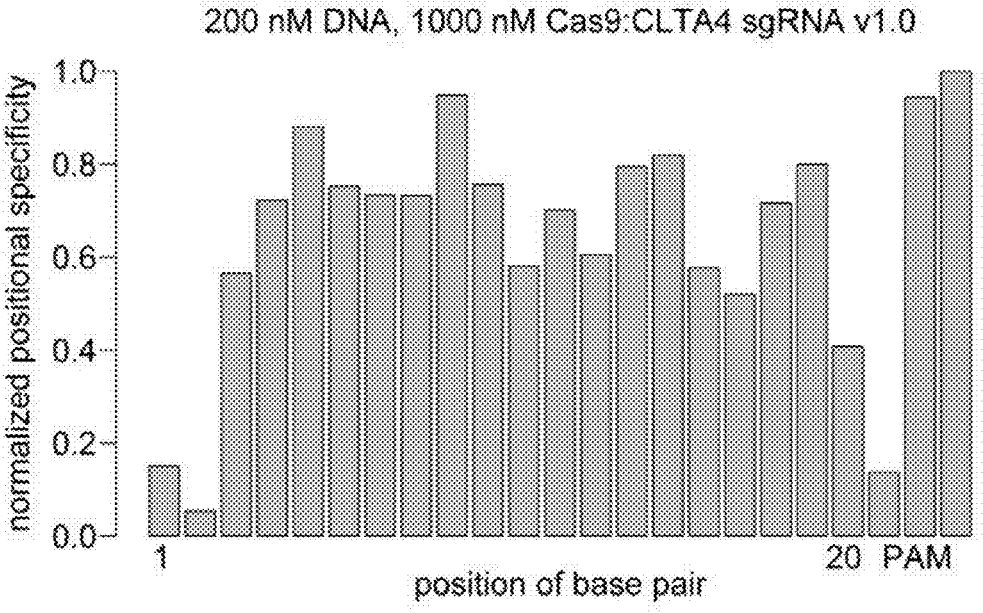


FIG. 19D

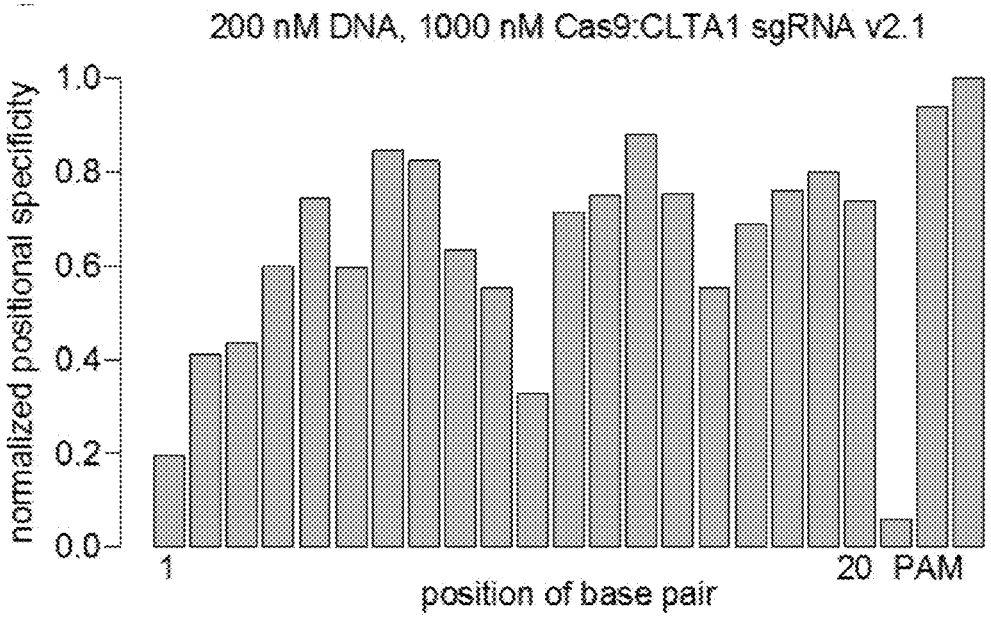


FIG. 20A

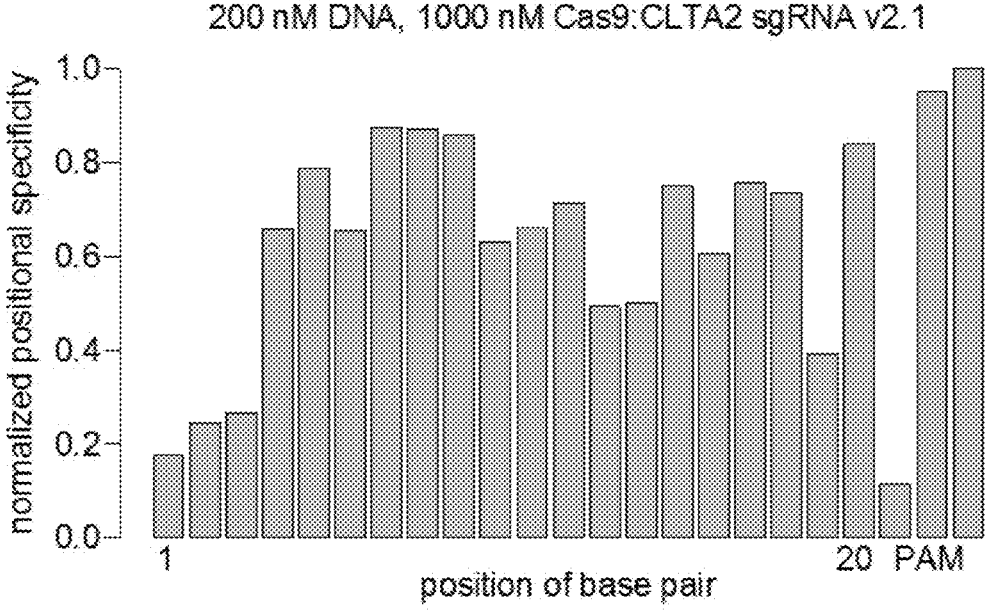


FIG. 20B

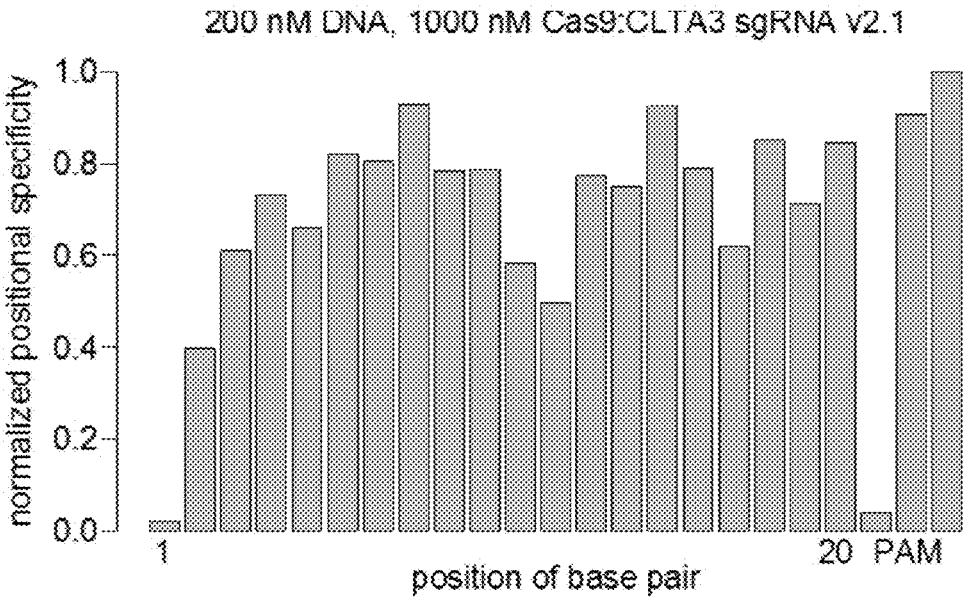


FIG. 20C

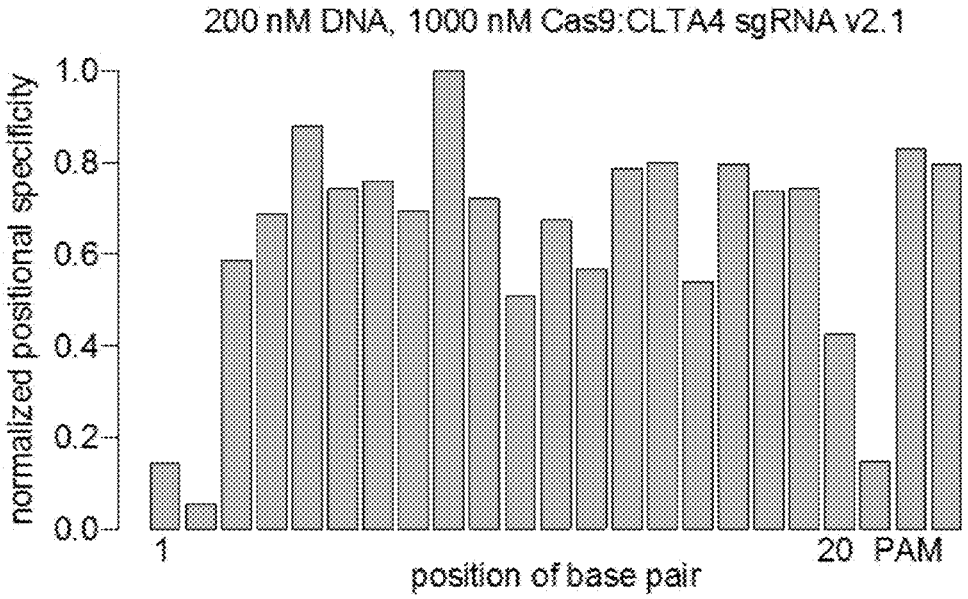


FIG. 20D

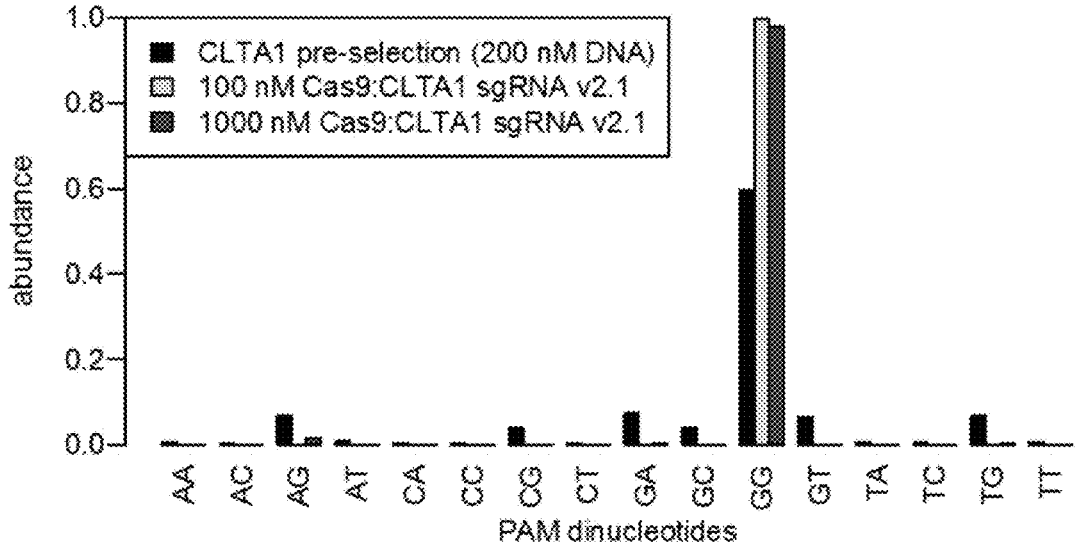


FIG. 21A

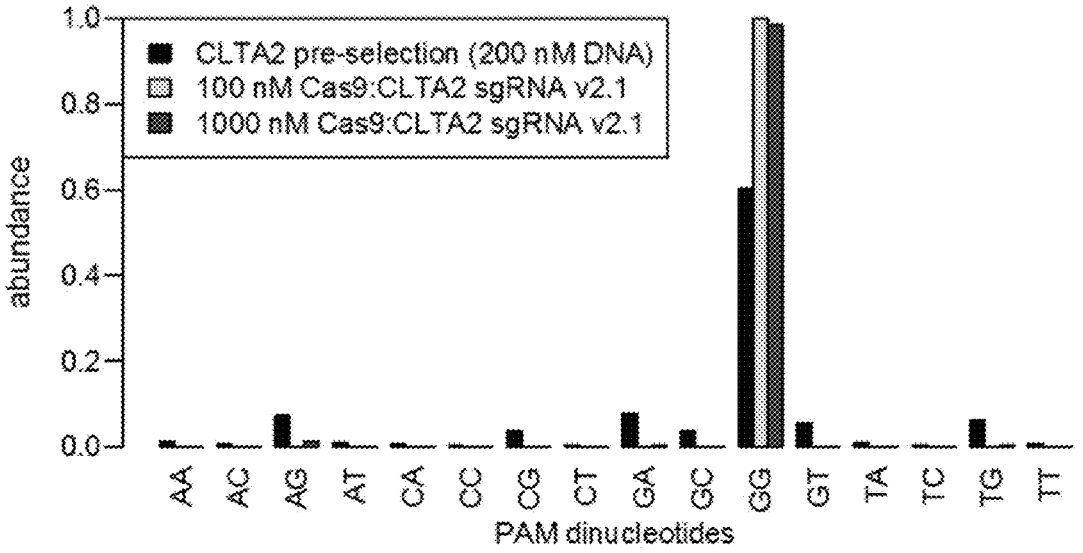


FIG. 21B

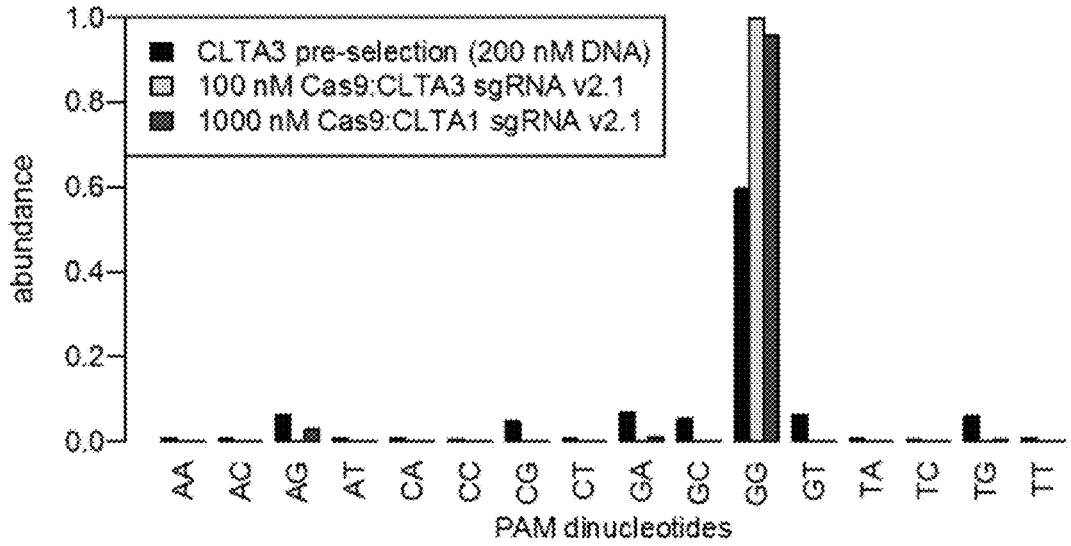


FIG. 21C

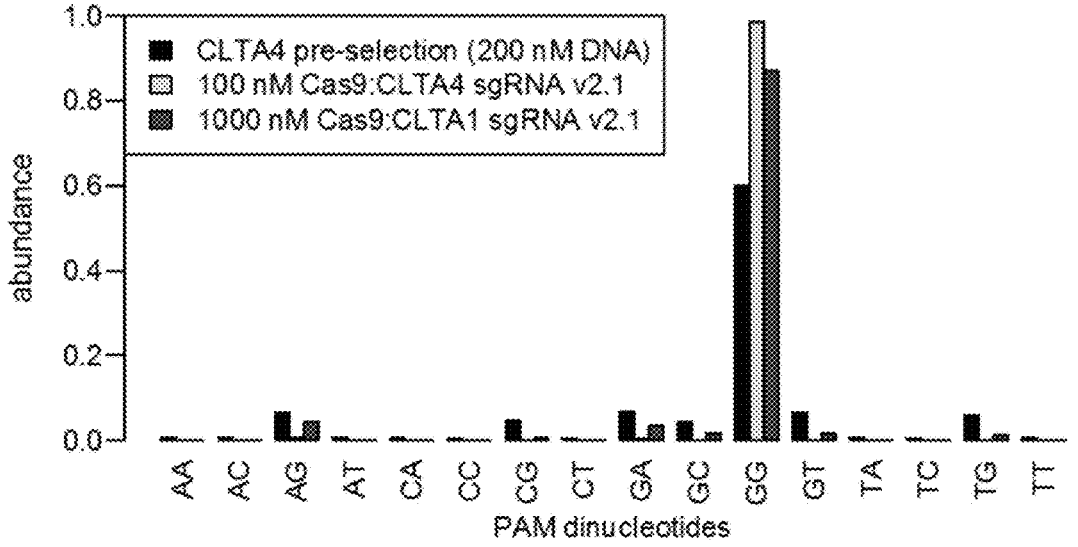


FIG. 21D

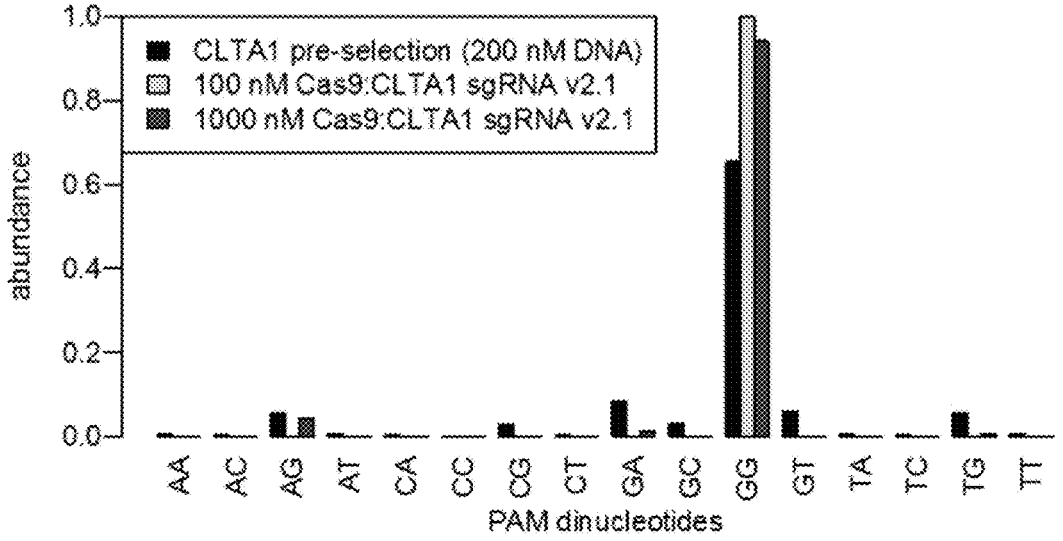


FIG. 22A

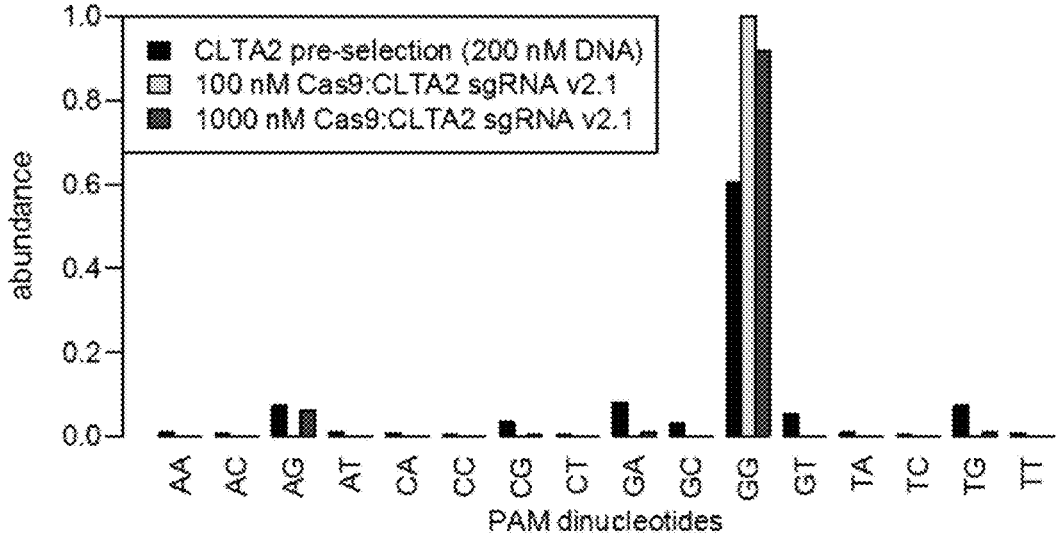


FIG. 22B

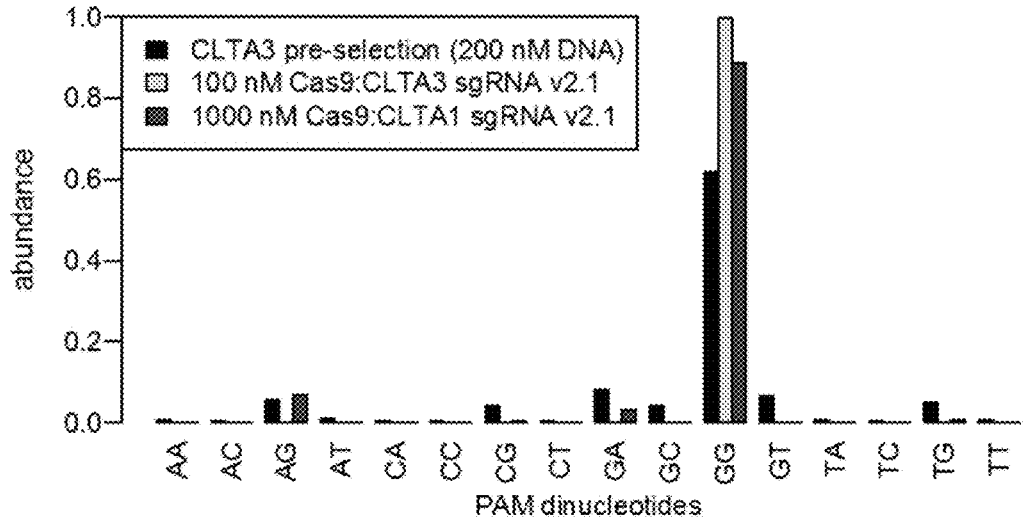


FIG. 22C

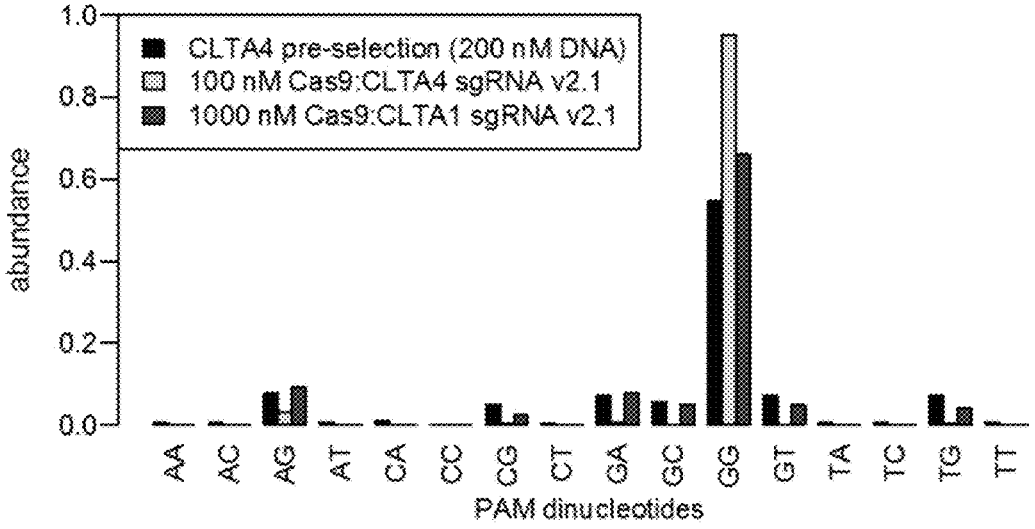


FIG. 22D

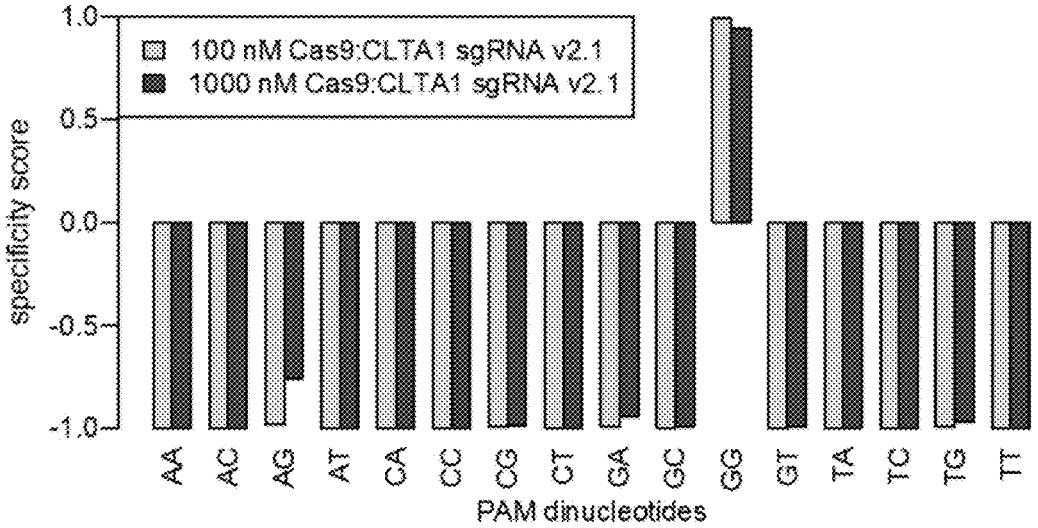


FIG. 23A

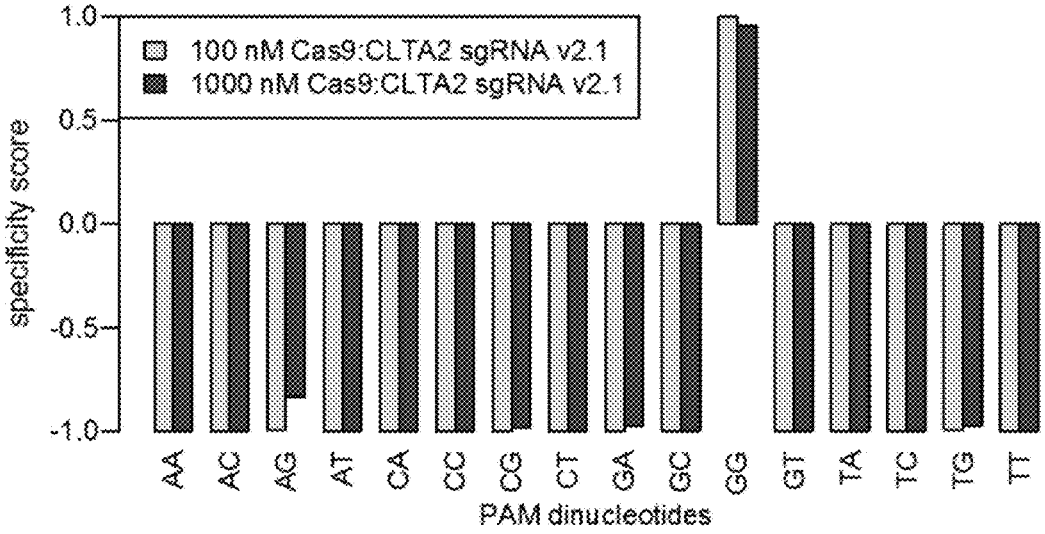


FIG. 23B

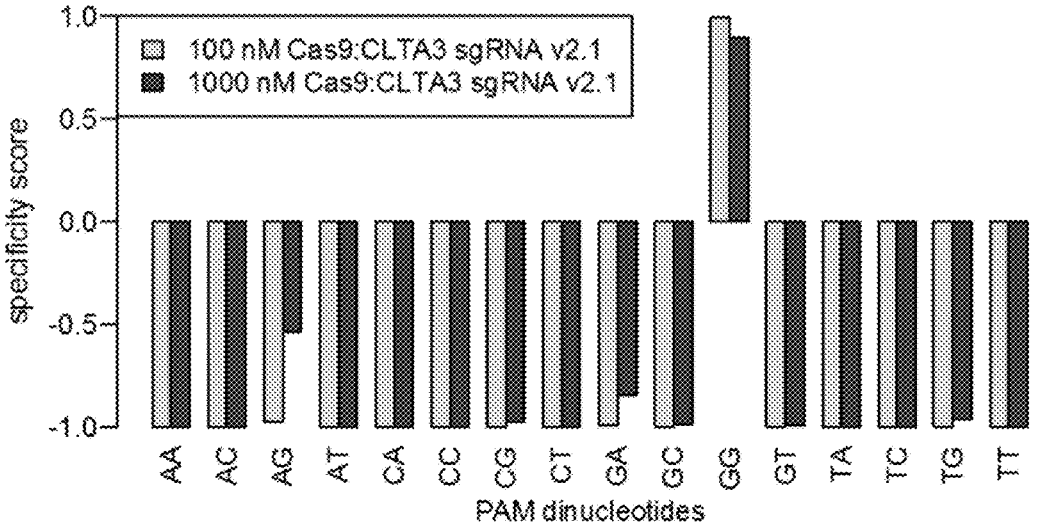


FIG. 23C

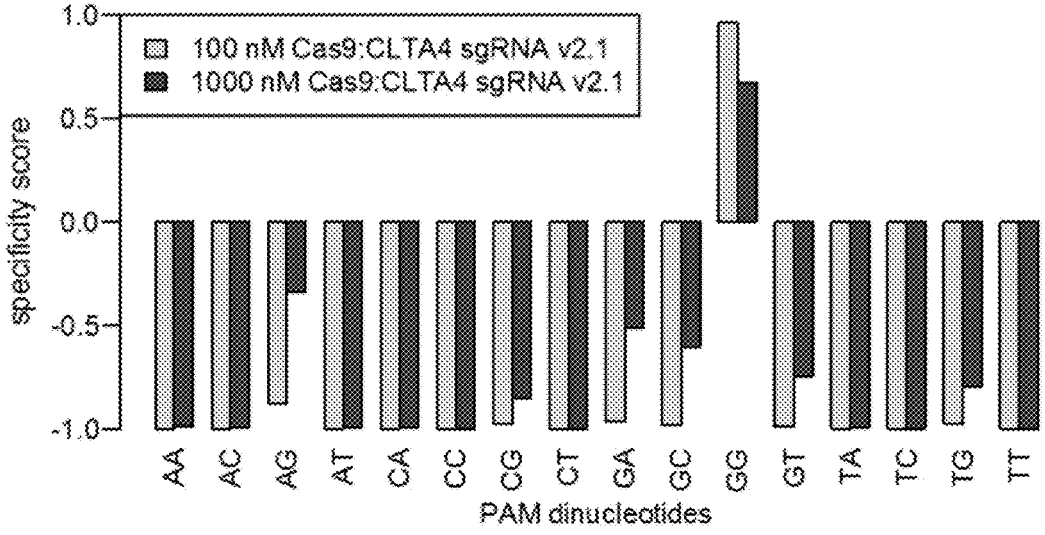


FIG. 23D

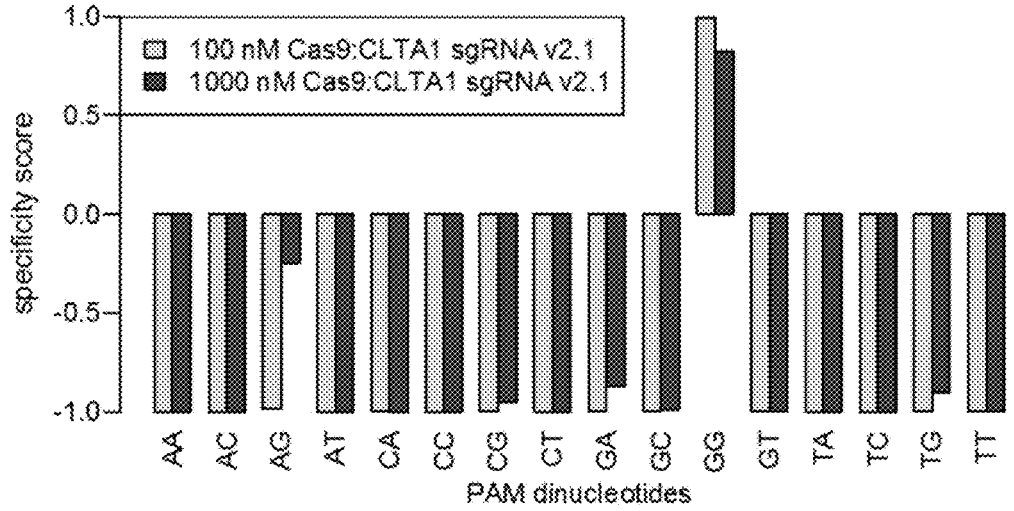


FIG. 24A

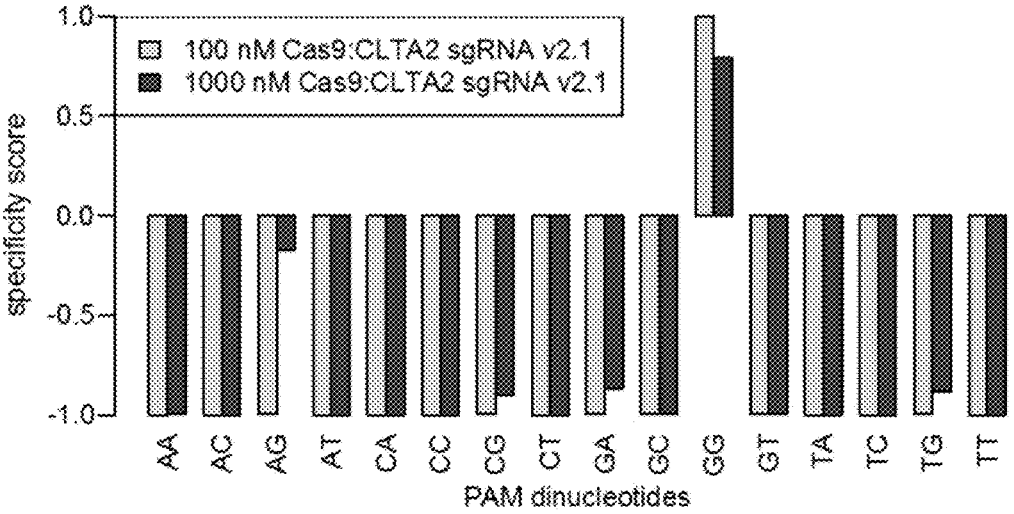


FIG. 24B

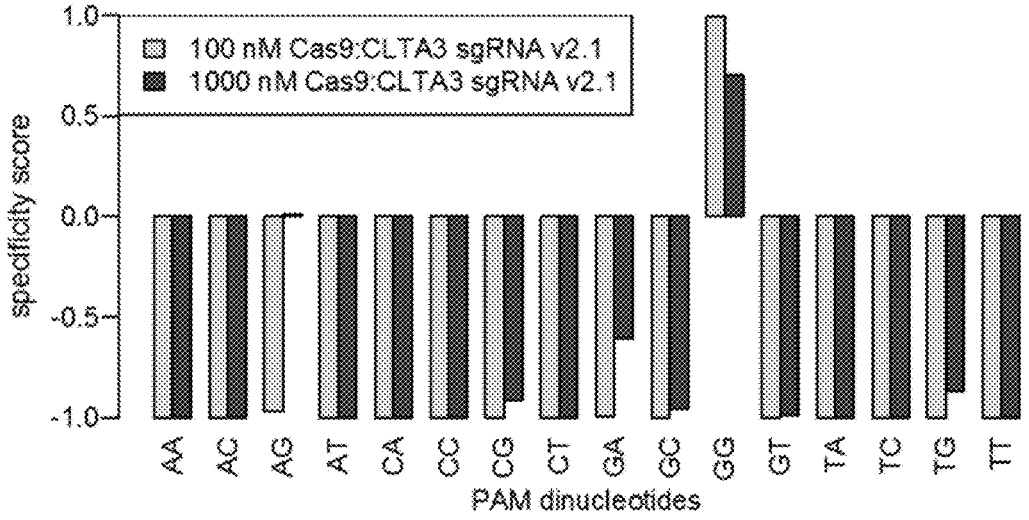


FIG. 24C

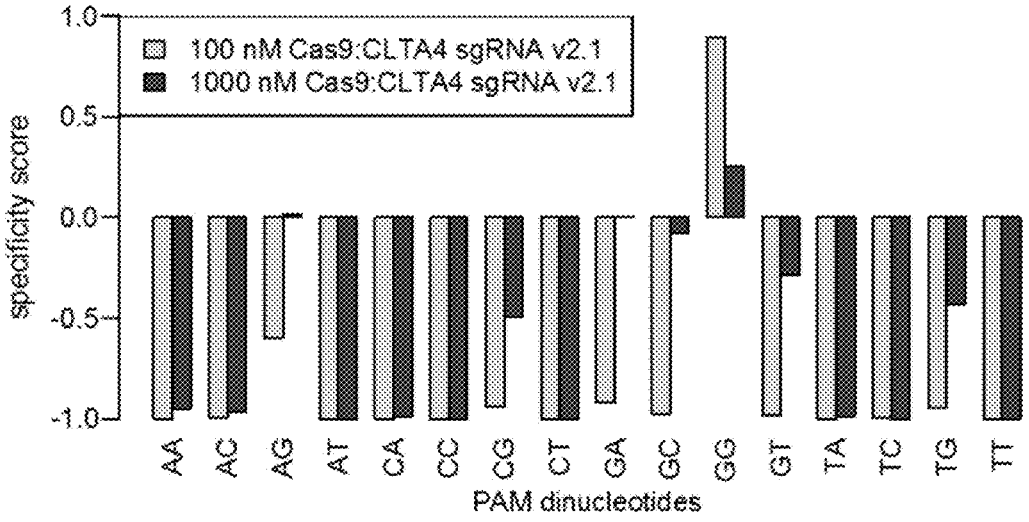


FIG. 24D

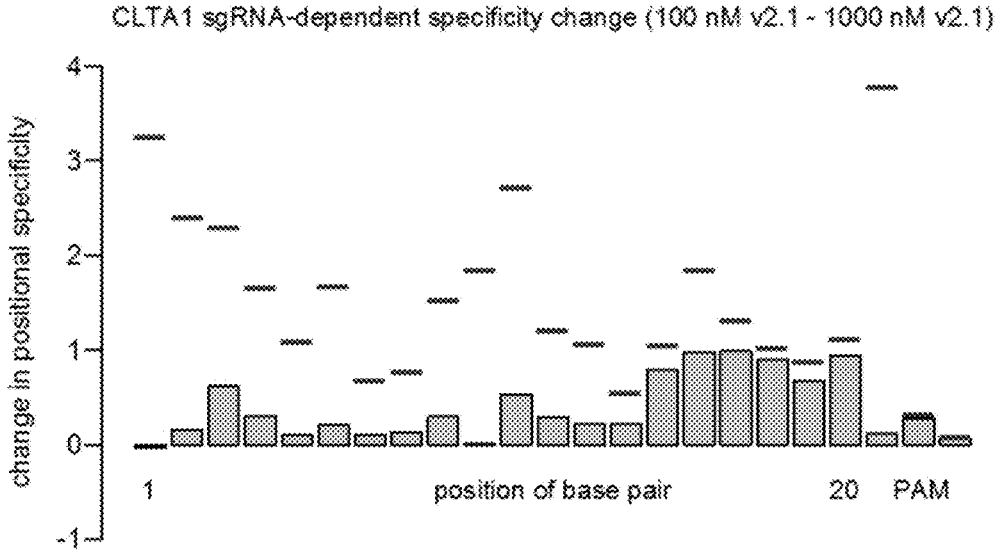


FIG. 25A

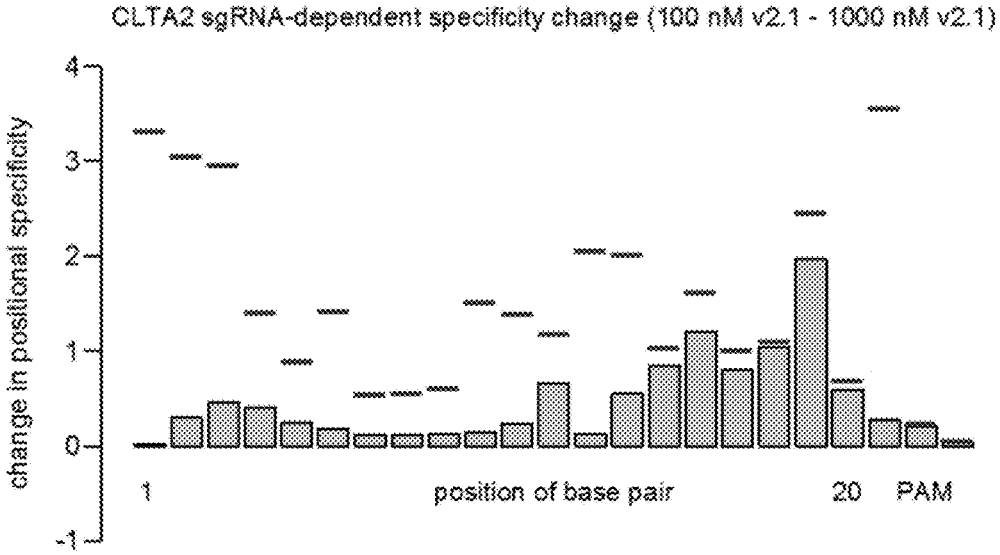


FIG. 25B

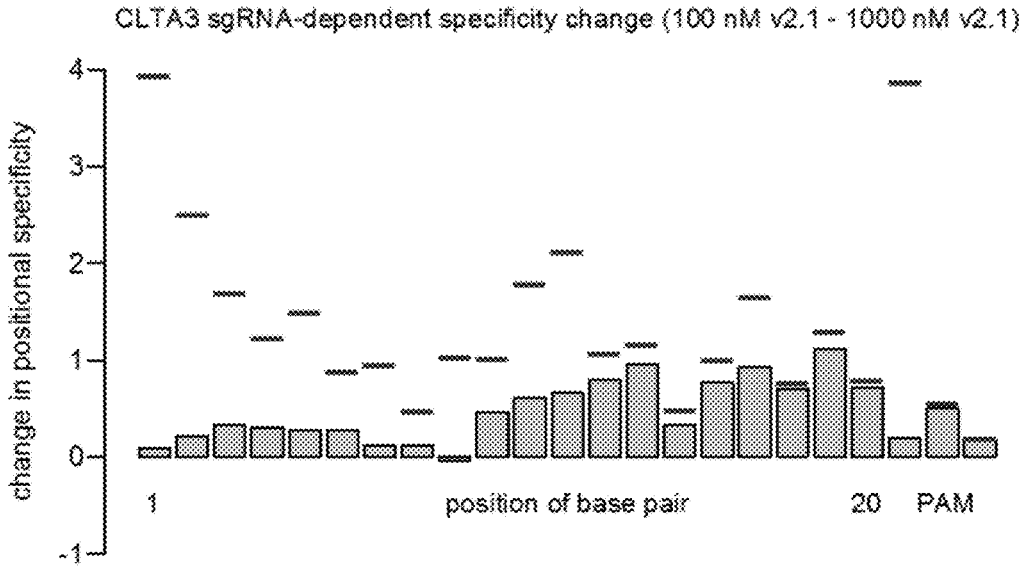


FIG. 25C

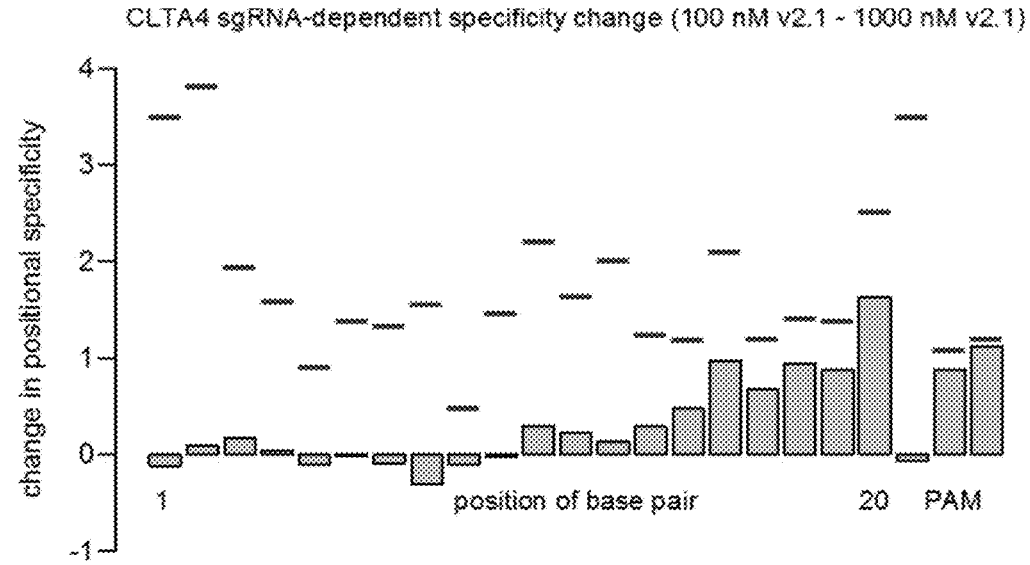


FIG. 25D

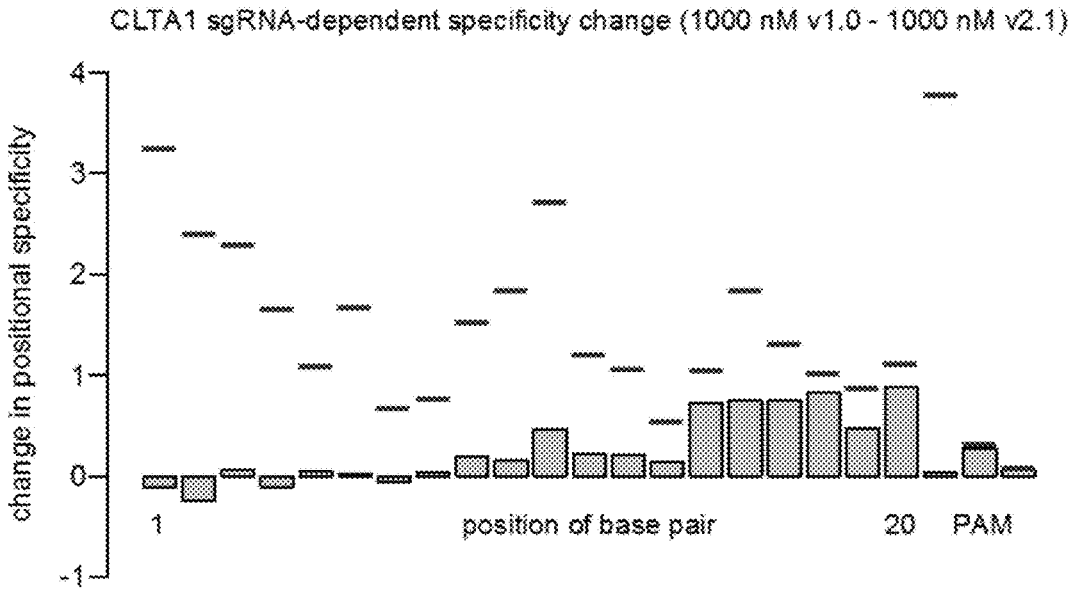


FIG. 26A

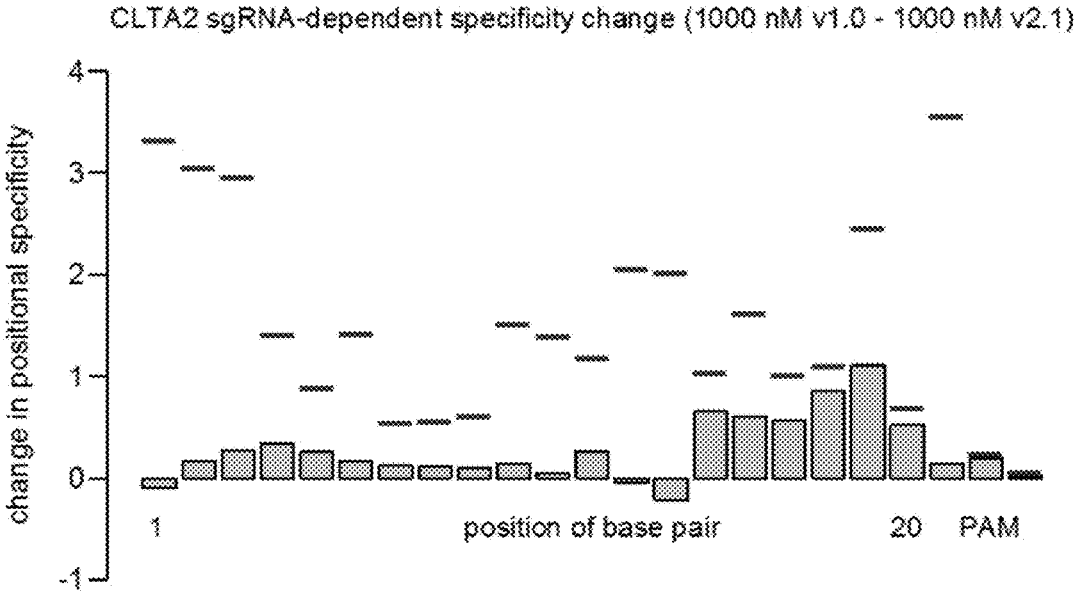


FIG. 26B

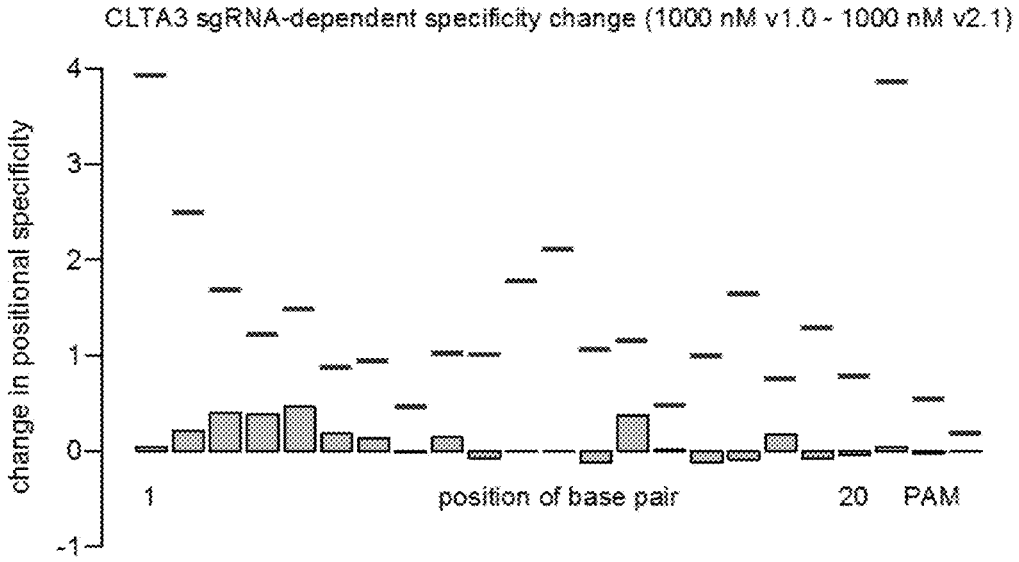


FIG. 26C

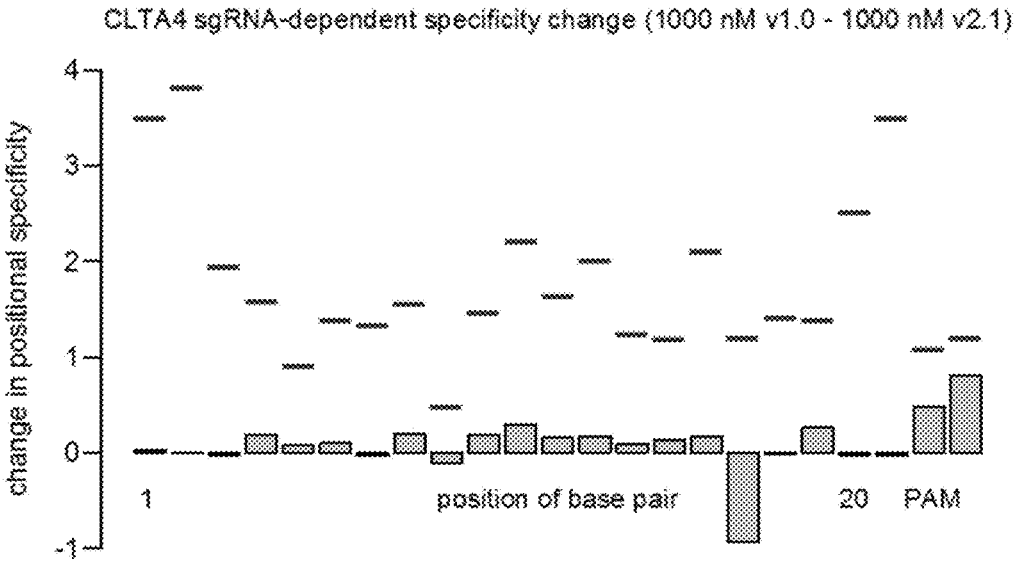
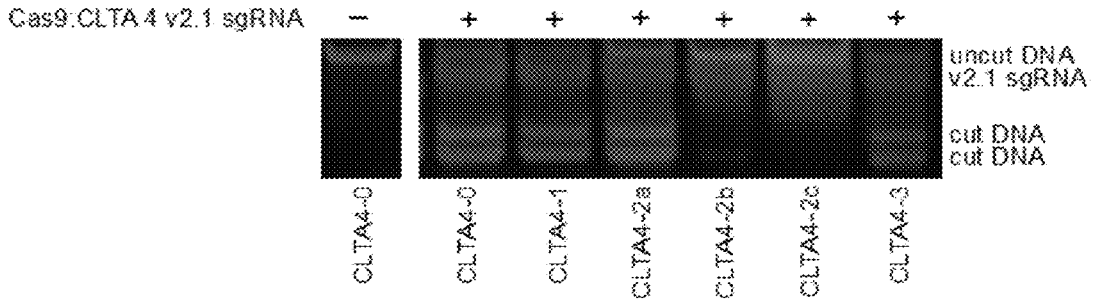


FIG. 26D



	sequence	*In vitro* selection enrichment value	% cut
CLTA4-0	GCAGATGTAGTGTTTCCACAGGG	7.9	85%
CLTA4-1	GaAGATGTAGTGTTTCCACAGGG	27.5	84%
CLTA4-2a	GaAGATGTAGTGTTTCCACtGGG	43.9	79%
CLTA4-2b	GCAGATGgAGgGTTTCCACAGGG	1.0	35%
CLTA4-2c	GCAGATGTAGTGTTaCCAgAGGG	0.064	none detected
CLTA4-3	GggGATGTAGTGTTTCCACtGGG	95.9	72%

FIG. 27

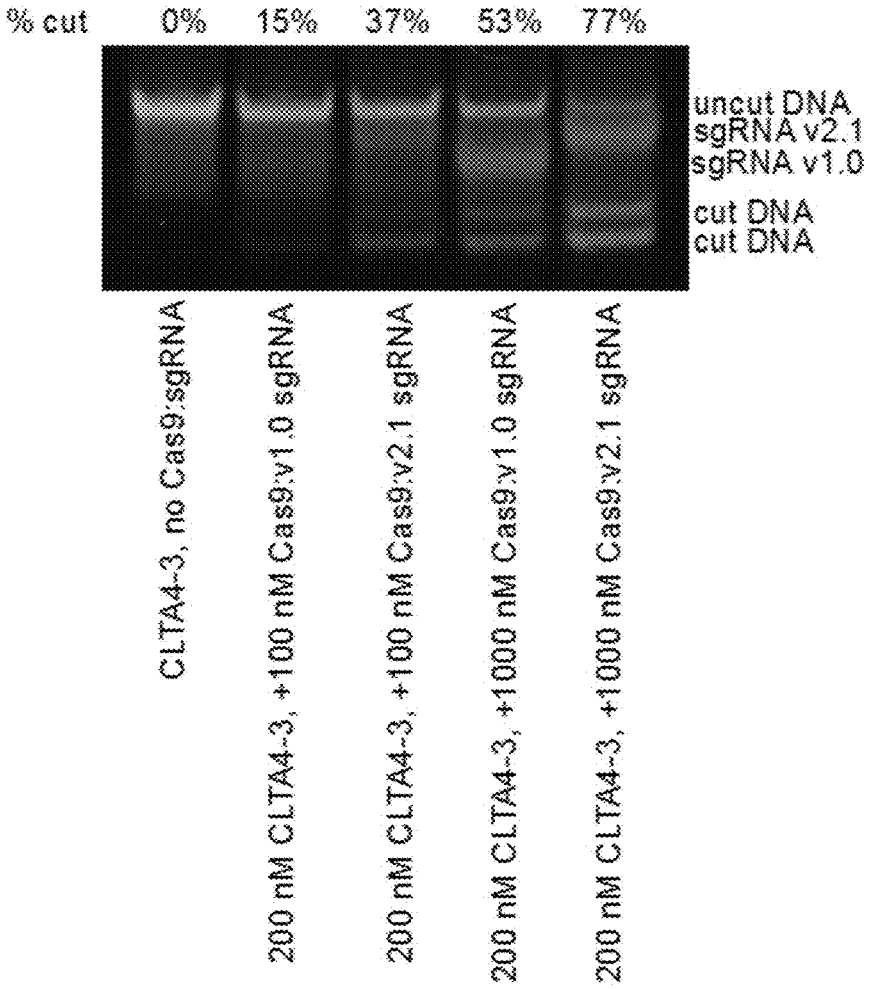

% cut
0%
15%
37%
53%
77%
uncut DNA
sgRNA v2.1
sgRNA v1.0
cut DNA
cut DNA
CLTA4-3, no Cas9:sgRNA
200 nM CLTA4-3, +100 nM Cas9:v1.0 sgRNA
200 nM CLTA4-3, +100 nM Cas9:v2.1 sgRNA
200 nM CLTA4-3, +1000 nM Cas9:v1.0 sgRNA
200 nM CLTA4-3, +1000 nM Cas9:v2.1 sgRNA

FIG. 28

METHODS FOR IDENTIFYING A TARGET SITE OF A CAS9 NUCLEASE

RELATED APPLICATION

This application claims priority under 35 U.S.C. §119(e) to U.S. provisional patent application, U.S. Ser. No. 61/864,289, filed Aug. 9, 2013, the entire contents of which are incorporated herein by reference.

GOVERNMENT SUPPORT

This invention was made with U.S. Government support under grant numbers HR0011-11-2-0003 and N66001-12-C-4207, awarded by the Defense Advanced Research Projects Agency. The U.S. Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Site-specific endonucleases theoretically allow for the targeted manipulation of a single site within a genome and are useful in the context of gene targeting as well as for therapeutic applications. In a variety of organisms, including mammals, site-specific endonucleases have been used for genome engineering by stimulating either non-homologous end joining or homologous recombination. In addition to providing powerful research tools, site-specific nucleases also have potential as gene therapy agents, and two site-specific endonucleases have recently entered clinical trials: one, CCR5-2246, targeting a human CCR-5 allele as part of an anti-HIV therapeutic approach (NCT00842634, NCT01044654, NCT01252641), and the other one, VF24684, targeting the human VEGF-A promoter as part of an anti-cancer therapeutic approach (NCT01082926).

Specific cleavage of the intended nuclease target site without or with only minimal off-target activity is a prerequisite for clinical applications of site-specific endonuclease, and also for high-efficiency genomic manipulations in basic research applications, as imperfect specificity of engineered site-specific binding domains has been linked to cellular toxicity and undesired alterations of genomic loci other than the intended target. Most nucleases available today, however, exhibit significant off-target activity, and thus may not be suitable for clinical applications. Technology for evaluating nuclease specificity and for engineering nucleases with improved specificity are therefore needed.

SUMMARY OF THE INVENTION

Some aspects of this disclosure are based on the recognition that the reported toxicity of some engineered site-specific endonucleases is based on off-target DNA cleavage, rather than on off-target binding alone. Some aspects of this disclosure provide strategies, compositions, systems, and methods to evaluate and characterize the sequence specificity of site-specific nucleases, for example, RNA-programmable endonucleases, such as Cas9 endonucleases, zinc finger nucleases (ZNFs), homing endonucleases, or transcriptional activator-like element nucleases (TALENs).

The strategies, methods, and reagents of the present disclosure represent, in some aspects, an improvement over previous methods for assaying nuclease specificity. For example, some previously reported methods for determining nuclease target site specificity profiles by screening libraries of nucleic acid molecules comprising candidate target sites relied on a "two-cut" in vitro selection method which requires indirect

reconstruction of target sites from sequences of two half-sites resulting from two adjacent cuts of the nuclease of a library member nucleic acid (see e.g., PCT Application WO 2013/066438; and Pattanayak, V., Ramirez, C. L., Joung, J. K. & Liu, D. R. Revealing off-target cleavage specificities of zinc-finger nucleases by in vitro selection. *Nature methods* 8, 765-770 (2011), the entire contents of each of which are incorporated herein by reference). In contrast to such "two-cut" strategies, the methods of the present disclosure utilize an optimized "one cut" screening strategy, which allows for the identification of library members that have been cut at least once by the nuclease. As explained in more detail elsewhere herein, the "one-cut" selection strategies provided herein are compatible with single end high-throughput sequencing methods and do not require computational reconstruction of cleaved target sites from cut half-sites, thus streamlining the nuclease profiling process.

Some aspects of this disclosure provide in vitro selection methods for evaluating the cleavage specificity of endonucleases and for selecting nucleases with a desired level of specificity. Such methods are useful, for example, for characterizing an endonuclease of interest and for identifying a nuclease exhibiting a desired level of specificity, for example, for identifying a highly specific endonuclease for clinical applications.

Some aspects of this disclosure provide methods of identifying suitable nuclease target sites that are sufficiently different from any other site within a genome to achieve specific cleavage by a given nuclease without any or at least minimal off-target cleavage. Such methods are useful for identifying candidate nuclease target sites that can be cleaved with high specificity on a genomic background, for example, when choosing a target site for genomic manipulation in vitro or in vivo.

Some aspects of this disclosure provide methods of evaluating, selecting, and/or designing site-specific nucleases with enhanced specificity as compared to current nucleases. For example, provided herein are methods that are useful for selecting and/or designing site-specific nucleases with minimal off-target cleavage activity, for example, by designing variant nucleases with binding domains having decreased binding affinity, by lowering the final concentration of the nuclease, by choosing target sites that differ by at least three base pairs from their closest sequence relatives in the genome, and, in the case of RNA-programmable nucleases, by selecting a guide RNA that results in the fewest off-target sites being bound and/or cut.

Compositions and kits useful in the practice of the methods described herein are also provided.

Some aspects of this disclosure provide methods for identifying a target site of a nuclease. In some embodiments, the method comprises (a) providing a nuclease that cuts a double-stranded nucleic acid target site, wherein cutting of the target site results in cut nucleic acid strands comprising a 5' phosphate moiety; (b) contacting the nuclease of (a) with a library of candidate nucleic acid molecules, wherein each nucleic acid molecule comprises a concatemer of a sequence comprising a candidate nuclease target site and a constant insert sequence, under conditions suitable for the nuclease to cut a candidate nucleic acid molecule comprising a target site of the nuclease; and (c) identifying nuclease target sites cut by the nuclease in (b) by determining the sequence of an uncut nuclease target site on the nucleic acid strand that was cut by the nuclease in step (b). In some embodiments, the nuclease creates blunt ends. In some embodiments, the nuclease creates a 5' overhang. In some embodiments, the determining of step (c) comprises ligating a first nucleic acid adapter to the 5'

end of a nucleic acid strand that was cut by the nuclease in step (b) via 5'-phosphate-dependent ligation. In some embodiments, the nucleic acid adapter is provided in double-stranded form. In some embodiments, the 5'-phosphate-dependent ligation is a blunt end ligation. In some embodiments, the method comprises filling in the 5'-overhang before ligating the first nucleic acid adapter to the nucleic acid strand that was cut by the nuclease. In some embodiments, the determining of step (c) further comprises amplifying a fragment of the concatemer cut by the nuclease that comprises an uncut target site via a PCR reaction using a PCR primer that hybridizes with the adapter and a PCR primer that hybridizes with the constant insert sequence. In some embodiments, the method further comprises enriching the amplified nucleic acid molecules for molecules comprising a single uncut target sequence. In some embodiments, the step of enriching comprises a size fractionation. In some embodiments, the determining of step (c) comprises sequencing the nucleic acid strand that was cut by the nuclease in step (b), or a copy thereof obtained via PCR. In some embodiments, the library of candidate nucleic acid molecules comprises at least 10^8, at least 10^9, at least 10^{10}, at least 10^{11}, or at least 10^{12} different candidate nuclease cleavage sites. In some embodiments, the nuclease is a therapeutic nuclease which cuts a specific nuclease target site in a gene associated with a disease. In some embodiments, the method further comprises determining a maximum concentration of the therapeutic nuclease at which the therapeutic nuclease cuts the specific nuclease target site, and does not cut more than 10, more than 5, more than 4, more than 3, more than 2, more than 1, or no additional nuclease target sites. In some embodiments, the method further comprises administering the therapeutic nuclease to a subject in an amount effective to generate a final concentration equal or lower than the maximum concentration. In some embodiments, the nuclease is an RNA-programmable nuclease that forms a complex with an RNA molecule, and wherein the nuclease:RNA complex specifically binds a nucleic acid sequence complementary to the sequence of the RNA molecule. In some embodiments, the RNA molecule is a single-guide RNA (sgRNA). In some embodiments, the sgRNA comprises 5-50 nucleotides, 10-30 nucleotides, 15-25 nucleotides, 18-22 nucleotides, 19-21 nucleotides, e.g., 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides. In some embodiments, the nuclease is a Cas9 nuclease. In some embodiments, the nuclease target site comprises a [sgRNA-complementary sequence]-[protospacer adjacent motif (PAM)] structure, and the nuclease cuts the target site within the sgRNA-complementary sequence. In some embodiments, the sgRNA-complementary sequence comprises 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides. In some embodiments, the nuclease comprises an unspecific nucleic acid cleavage domain. In some embodiments, the nuclease comprises a FokI cleavage domain. In some embodiments, the nuclease comprises a nucleic acid cleavage domain that cleaves a target sequence upon cleavage domain dimerization. In some embodiments, the nuclease comprises a binding domain that specifically binds a nucleic acid sequence. In some embodiments, the binding domain comprises a zinc finger. In some embodiments, the binding domain comprises at least 2, at least 3, at least 4, or at least 5 zinc fingers. In some embodiments, the nuclease is a Zinc Finger Nuclease. In some embodiments, the binding domain comprises a Transcriptional Activator-Like Element. In some embodiments, the nuclease is a Transcriptional Activator-Like Element Nuclease (TALEN). In some embodiments, the nuclease is an organic compound. In some embodiments, the nuclease comprises an enediyne functional group. In some embodiments, the nuclease is an antibiotic. In some embodiments, the compound is dynemicin, neocarzinostatin, calicheamicin, esperamicin, bleomycin, or a derivative thereof. In some embodiments, the nuclease is a homing endonuclease.

Some aspects of this disclosure provide libraries of nucleic acid molecules, in which each nucleic acid molecule comprises a concatemer of a sequence comprising a candidate nuclease target site and a constant insert sequence of 10-100 nucleotides. In some embodiments, the constant insert sequence is at least 15, at least 20, at least 25, at least 30, at least 35, at least 40, at least 45, at least 50, at least 55, at least 60, at least 65, at least 70, at least 75, at least 80, or at least 95 nucleotides long. In some embodiments, the constant insert sequence is not more than 15, not more than 20, not more than 25, not more than 30, not more than 35, not more than 40, not more than 45, not more than 50, not more than 55, not more than 60, not more than 65, not more than 70, not more than 75, not more than 80, or not more than 95 nucleotides long. In some embodiments, the candidate nuclease target sites are sites that can be cleaved by an RNA-programmable nuclease, a Zinc Finger Nuclease (ZFN), a Transcription Activator-Like Effector Nuclease (TALEN), a homing endonuclease, an organic compound nuclease, or an enediyne antibiotic (e.g., dynemicin, neocarzinostatin, calicheamicin, esperamicin, bleomycin). In some embodiments, the candidate nuclease target site can be cleaved by a Cas9 nuclease. In some embodiments, the library comprises at least 10^5, at least 10^6, at least 10^7, at least 10^8, at least 10^9, at least 10^{10}, at least 10^{11}, or at least 10^{12} different candidate nuclease target sites. In some embodiments, the library comprises nucleic acid molecules of a molecular weight of at least 0.5 kDa, at least 1 kDa, at least 2 kDa, at least 3 kDa, at least 4 kDa, at least 5 kDa, at least 6 kDa, at least 7 kDa, at least 8 kDa, at least 9 kDa, at least 10 kDa, at least 12 kDa, or at least 15 kDa. In some embodiments, the library comprises candidate nuclease target sites that are variations of a known target site of a nuclease of interest. In some embodiments, the variations of a known nuclease target site comprise 10 or fewer, 9 or fewer, 8 or fewer, 7 or fewer, 6 or fewer, 5 or fewer, 4 or fewer, 3 or fewer, or 2 or fewer mutations as compared to a known nuclease target site. In some embodiments, the variations differ from the known target site of the nuclease of interest by more than 5%, more than 10%, more than 15%, more than 20%, more than 25%, or more than 30% on average, distributed binomially. In some embodiments, the variations differ from the known target site by no more than 10%, no more than 15%, no more than 20%, no more than 25%, nor more than 30%, no more than 40%, or no more than 50% on average, distributed binomially. In some embodiments, the nuclease of interest is a Cas9 nuclease, a zinc finger nuclease, a TALEN, a homing endonuclease, an organic compound nuclease, or an enediyne antibiotic (e.g., dynemicin, neocarzinostatin, calicheamicin, esperamicin, bleomycin). In some embodiments, the candidate nuclease target sites are Cas9 nuclease target sites that comprise a [sgRNA-complementary sequence]-[PAM] structure, wherein the sgRNA-complementary sequence comprises 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides.

Some aspects of this disclosure provide methods for selecting a nuclease that specifically cuts a consensus target site from a plurality of nucleases. In some embodiments, the method comprises (a) providing a plurality of candidate nucleases that cut the same consensus sequence; (b) for each of the candidate nucleases of step (a), identifying a nuclease target site cleaved by the candidate nuclease that differ from

the consensus target site using a method provided herein; (c) selecting a nuclease based on the nuclease target site(s) identified in step (b). In some embodiments, the nuclease selected in step (c) is the nuclease that cleaves the consensus target site with the highest specificity. In some embodiments, the nuclease that cleaves the consensus target site with the highest specificity is the candidate nuclease that cleaves the lowest number of target sites that differ from the consensus site. In some embodiments, the candidate nuclease that cleaves the consensus target site with the highest specificity is the candidate nuclease that cleaves the lowest number of target sites that are different from the consensus site in the context of a target genome. In some embodiments, the candidate nuclease selected in step (c) is a nuclease that does not cleave any target site other than the consensus target site. In some embodiments, the candidate nuclease selected in step (c) is a nuclease that does not cleave any target site other than the consensus target site within the genome of a subject at a therapeutically effective concentration of the nuclease. In some embodiments, the method further comprises contacting a genome with the nuclease selected in step (c). In some embodiments, the genome is a vertebrate, mammalian, human, non-human primate, rodent, mouse, rat, hamster, goat, sheep, cattle, dog, cat, reptile, amphibian, fish, nematode, insect, or fly genome. In some embodiments, the genome is within a living cell. In some embodiments, the genome is within a subject. In some embodiments, the consensus target site is within an allele that is associated with a disease or disorder. In some embodiments, cleavage of the consensus target site results in treatment or prevention of a disease or disorder, e.g., amelioration or prevention of at least one sign and/or symptom of the disease or disorder. In some embodiments, cleavage of the consensus target site results in the alleviation of a sign and/or symptom of the disease or disorder. In some embodiments, cleavage of the consensus target site results in the prevention of the disease or disorder. In some embodiments, the disease is HIV/AIDS. In some embodiments, the allele is a CCR5 allele. In some embodiments, the disease is a proliferative disease. In some embodiments, the disease is cancer. In some embodiments, the allele is a VEGFA allele.

Some aspects of this disclosure provide isolated nucleases that have been selected according to a method provided herein. In some embodiments, the nuclease has been engineered to cleave a target site within a genome. In some embodiments, the nuclease is a Cas9 nuclease comprising an sgRNA that is complementary to the target site within the genome. In some embodiments, the nuclease is a Zinc Finger Nuclease (ZFN) or a Transcription Activator-Like Effector Nuclease (TALEN), a homing endonuclease, or an organic compound nuclease (e.g., an enediyne, an antibiotic nuclease, dynemicin, neocarzinostatin, calicheamicin, esperamicin, bleomycin, or a derivative thereof). In some embodiments, the nuclease has been selected based on cutting no other candidate target site, not more than one candidate target site, not more than two candidate target sites, not more than three candidate target sites, not more than four candidate target sites, not more than five candidate target sites, not more than six candidate target sites, not more than seven candidate target sites, not more than eight candidate target sites, not more than eight candidate target sites, not more than nine candidate target sites, or not more than ten candidate target sites in addition to its known nuclease target site.

Some aspects of this disclosure provide kits comprising a library of nucleic acid molecules comprising candidate nuclease target sites as provided herein. Some aspects of this disclosure provide kits comprising an isolated nuclease as provided herein. In some embodiments, the nuclease is a Cas9 nuclease. In some embodiments, the kit further comprises a nucleic acid molecule comprising a target site of the isolated nuclease. In some embodiments, the kit comprises an excipient and instructions for contacting the nuclease with the excipient to generate a composition suitable for contacting a nucleic acid with the nuclease. In some embodiments, the composition is suitable for contacting a nucleic acid within a genome. In some embodiments, the composition is suitable for contacting a nucleic acid within a cell. In some embodiments, the composition is suitable for contacting a nucleic acid within a subject. In some embodiments, the excipient is a pharmaceutically acceptable excipient.

Some aspects of this disclosure provide pharmaceutical compositions that are suitable for administration to a subject. In some embodiments, the composition comprises an isolated nuclease as provided herein. In some embodiments, the composition comprises a nucleic acid encoding such a nuclease. In some embodiments, the composition comprises a pharmaceutically acceptable excipient.

Other advantages, features, and uses of the invention will be apparent from the detailed description of certain non-limiting embodiments of the invention; the drawings, which are schematic and not intended to be drawn to scale; and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. **1**A-B. In vitro selection overview. (a) Cas9 complexed with a short guide RNA (sgRNA) recognizes ~20 bases of a target DNA substrate that is complementary to the sgRNA sequence and cleaves both DNA strands. The white triangles represent cleavage locations. (b) A modified version of our previously described in vitro selection was used to comprehensively profile Cas9 specificity. A concatemeric pre-selection DNA library in which each molecule contains one of 10^{12} distinct variants of a target DNA sequence (white rectangles) was generated from synthetic DNA oligonucleotides by ligation and rolling-circle amplification. This library was incubated with a Cas9:sgRNA complex of interest. Cleaved library members contain 5' phosphate groups (circles) and therefore are substrates for adapter ligation and PCR. The resulting amplicons were subjected to high-throughput DNA sequencing and computational analysis.

FIGS. **2**A-H. In vitro selection results for Cas9:CLTA1 sgRNA. Heat maps[21] show the specificity profiles of Cas9:CLTA1 sgRNA v2.1 under enzyme-limiting conditions (a, b), Cas9:CLTA1 sgRNA v1.0 under enzyme-saturating conditions (c, d), and Cas9:CLTA1 sgRNA v2.1 under enzyme-saturating conditions (e, f). Heat maps show all post-selection sequences (a, c, e) or only those sequences containing a single mutation in the 20-base pair sgRNA-specified target site and two-base pair PAM (b, d, f). Specificity scores of 1.0 and −1.0 corresponds to 100% enrichment for and against, respectively, a particular base pair at a particular position. Black boxes denote the intended target nucleotides. (g) Effect of Cas9:sgRNA concentration on specificity. Positional specificity changes between enzyme-limiting (200 nM DNA, 100 nM Cas9:sgRNA v2.1) and enzyme-saturating (200 nM DNA, 1000 nM Cas9:sgRNA v2.1) conditions, normalized to the maximum possible change in positional specificity, are shown for CLTA1. (h) Effect of sgRNA architecture on specificity. Positional specificity changes between sgRNA v1.0 and sgRNA v2.1 under enzyme-saturating conditions, normalized to the maximum possible change in positional specificity, are shown for CLTA1. See FIGS. **6**-**8**, **25**, and **26** for corresponding data for CLTA2, CLTA3, and CLTA4.

FIGS. **3**A-D. Target sites profiled in this study. (A) The 5' end of the sgRNA has 20 nucleotides that are complementary to the target site. The target site contains an NGG motif (PAM) adjacent to the region of RNA:DNA complementarity. (B) Four human clathrin gene (CLTA) target sites are shown. Sequences correspond, from top to bottom, to SEQ ID NOs: 2-7, respectively. (C, D) Four human clathrin gene (CLTA) target sites are shown with sgRNAs. sgRNA v1.0 is shorter than sgRNA v2.1. The PAM is shown for each site. The non-PAM end of the target site corresponds to the 5' end of the sgRNA. Sequences in FIG. **3**C correspond, from top to bottom, to SEQ ID NOs: 8-19, respectively. Sequences in FIG. **3**D correspond, from top to bottom, to SEQ ID NOs: 20-31, respectively.

FIG. **4**. Cas9:guide RNA cleavage of on-target DNA sequences in vitro. Discrete DNA cleavage assays on an approximately 1-kb linear substrate were performed with 200 nM on-target site and 100 nM Cas9:v1.0 sgRNA, 100 nM Cas9:v2.1 sgRNA, 1000 nM Cas9:v1.0 sgRNA, and 1000 nM Cas9:v2.1 sgRNA for each of four CLTA target sites. For CLTA1, CLTA2, and CLTA4, Cas9:v2.1 sgRNA shows higher activity than Cas9:v1.0 sgRNA. For CLTA3, the activities of the Cas9:v1.0 sgRNA and Cas9:v2.1 sgRNA were comparable.

FIGS. **5**A-E. In vitro selection results for four target sites. In vitro selections were performed on 200 nM pre-selection library with 100 nM Cas9:sgRNA v2.1, 1000 nM Cas9:sgRNA v1.0, or 1000 nM Cas9:sgRNA v2.1. (A) Post-selection PCR products are shown for the 12 selections performed. DNA containing 1.5 repeats were quantified for each selection and pooled in equimolar amounts before gel purification and sequencing. (B-E) Distributions of mutations are shown for pre-selection (black) and post-selection libraries (colored). The post-selection libraries are enriched for sequences with fewer mutations than the pre-selection libraries. Mutations are counted from among the 20 base pairs specified by the sgRNA and the two-base pair PAM. P-values are <0.01 for all pairwise comparisons between distributions in each panel. P-values were calculated using t-tests, assuming unequal size and unequal variance.

FIGS. **6**A-F. In vitro selection results for Cas9:CLTA2 sgRNA. Heat maps[24] show the specificity profiles of Cas9:CLTA2 sgRNA v2.1 under enzyme-limiting conditions (A, B), Cas9:CLTA2 sgRNA v1.0 under enzyme-excess conditions (C, D), and Cas9:CLTA2 sgRNA v2.1 under enzyme-excess conditions (E, F). Heat maps show all post-selection sequences (A, C, E) or only those sequences containing a single mutation in the 20-base pair sgRNA-specified target site and two-base pair PAM (B, D, F). Specificity scores of 1.0 and −1.0 corresponds to 100% enrichment for and against, respectively, a particular base pair at a particular position. Black boxes denote the intended target nucleotides.

FIGS. **7**A-F. In vitro selection results for Cas9:CLTA3 sgRNA. Heat maps[24] show the specificity profiles of Cas9:CLTA3 sgRNA v2.1 under enzyme-limiting conditions (A, B), Cas9:CLTA3 sgRNA v1.0 under enzyme-excess conditions (C, D), and Cas9:CLTA3 sgRNA v2.1 under enzyme-saturating conditions (E, F). Heat maps show all post-selection sequences (A, C, E) or only those sequences containing a single mutation in the 20-base pair sgRNA-specified target site and two-base pair PAM (B, D, F). Specificity scores of 1.0 and −1.0 corresponds to 100% enrichment for and against, respectively, a particular base pair at a particular position. Black boxes denote the intended target nucleotides.

FIGS. **8**A-F. In vitro selection results for Cas9:CLTA4 sgRNA. Heat maps[24] show the specificity profiles of Cas9:CLTA4 sgRNA v2.1 under enzyme-limiting conditions (A, B), Cas9:CLTA4 sgRNA v1.0 under enzyme-excess conditions (C, D), and Cas9:CLTA4 sgRNA v2.1 under enzyme-saturating conditions (E, F). Heat maps show all post-selection sequences (A, C, E) or only those sequences containing a single mutation in the 20-base pair sgRNA-specified target site and two-base pair PAM (B, D, F). Specificity scores of 1.0 and −1.0 corresponds to 100% enrichment for and against, respectively, a particular base pair at a particular position. Black boxes denote the intended target nucleotides.

FIGS. **9**A-D. In vitro selection results as sequence logos. Information content is plotted[25] for each target site position (1-20) specified by CLTA1 (A), CLTA2 (B), CLTA3 (C), and CLTA4 (D) sgRNA v2.1 under enzyme-limiting conditions. Positions in the PAM are labelled "P1," "P2," and "P3." Information content is plotted in bits. 2.0 bits indicates absolute specificity and 0 bits indicates no specificity.

FIGS. **10**A-L. Tolerance of mutations distal to the PAM for CLTA1. The maximum specificity scores at each position are shown for the Cas9:CLTA1 v2.1 sgRNA selections when considering only those sequences with on-target base pairs in gray, while allowing mutations in the first 1-12 base pairs (a-l). The positions that are not constrained to on-target base pairs are indicated by dark bars. Higher specificity score values indicate higher specificity at a given position. The positions that were not allowed to contain any mutations (gray) were plotted with a specificity score of +1. For all panels, specificity scores were calculated from pre-selection library sequences and post-selection library sequences with an $n \geq 5{,}130$ and $n \geq 74{,}538$, respectively.

FIGS. **11**A-L. Tolerance of mutations distal to the PAM for CLTA2. The maximum specificity scores at each position are shown for the Cas9:CLTA2 v2.1 sgRNA selections when considering only those sequences with on-target base pairs in gray, while allowing mutations in the first 1-12 base pairs (a-l). The positions that are not constrained to on-target base pairs are indicated by dark bars. Higher specificity score values indicate higher specificity at a given position. The positions that were not allowed to contain any mutations (gray) were plotted with a specificity score of +1. For all panels, specificity scores were calculated from pre-selection library sequences and post-selection library sequences with an $n \geq 3{,}190$ and $n \geq 25{,}365$, respectively.

FIGS. **12**A-L. Tolerance of mutations distal to the PAM for CLTA3. The maximum specificity scores at each position are shown for the Cas9:CLTA3 v2.1 sgRNA selections when considering only those sequences with on-target base pairs in gray, while allowing mutations in the first 1-12 base pairs (a-l). The positions that are not constrained to on-target base pairs are indicated by dark bars. Higher specificity score values indicate higher specificity at a given position. The positions that were not allowed to contain any mutations (gray) were plotted with a specificity score of +1. For all panels, specificity scores were calculated from pre-selection library sequences and post-selection library sequences with an $n \geq 5{,}604$ and $n \geq 158{,}424$, respectively.

FIGS. **13**A-I. Tolerance of mutations distal to the PAM for CLTA4. The maximum specificity scores at each position are shown for the Cas9:CLTA4 v2.1 sgRNA selections when considering only those sequences with on-target base pairs in gray, while allowing mutations in the first 1-12 base pairs (a-i). The positions that are not constrained to on-target base pairs are indicated by dark bars. Higher specificity score values indicate higher specificity at a given position. The positions that were not allowed to contain any mutations (gray) were plotted with a specificity score of +1. For all panels, specificity scores were calculated from pre-selection

library sequences and post-selection library sequences with an n≥2,323 and n≥21,819, respectively.

FIGS. **14**A-L. Tolerance of mutations distal to the PAM in CLTA1 target sites. Distributions of mutations are shown for in vitro selection on 200 nM pre-selection library with 1000 nM Cas9:CLTA1 sgRNA v2.1. The number of mutations shown are in a 1-12 base pair target site subsequence farthest from the PAM (a-l) when the rest of the target site, including the PAM, contains only on-target base pairs. The pre-selection and post-selection distributions are similar for up to three base pairs, demonstrating tolerance for target sites with mutations in the three base pairs farthest from the PAM when the rest of the target sites have optimal interactions with the Cas9:sgRNA. For all panels, graphs were generated from pre-selection library sequences and post-selection library sequences with an n≥5,130 and n≥74,538, respectively.

FIGS. **15**A-L. Tolerance of mutations distal to the PAM in CLTA2 target sites. Distributions of mutations are shown for in vitro selection on 200 nM pre-selection library with 1000 nM Cas9:CLTA2 sgRNA v2.1. The number of mutations shown are in a 1-12 base pair target site subsequence farthest from the PAM (a-l) when the rest of the target site, including the PAM, contains only on-target base pairs. The pre-selection and post-selection distributions are similar for up to three base pairs, demonstrating tolerance for target sites with mutations in the three base pairs farthest from the PAM when the rest of the target sites have optimal interactions with the Cas9:sgRNA. For all panels, graphs were generated from pre-selection library sequences and post-selection library sequences with an n≥3,190 and n≥21,265, respectively.

FIGS. **16**A-L. Tolerance of mutations distal to PAM in CLTA3 target sites. Distributions of mutations are shown for in vitro selection on 200 nM pre-selection library with 1000 nM Cas9:CLTA3 sgRNA v2.1. The number of mutations shown are in a 1-12 base pair target site subsequence farthest from the PAM (a-l) when the rest of the target site, including the PAM, contains only on-target base pairs. The pre-selection and post-selection distributions are similar for up to three base pairs, demonstrating tolerance for target sites with mutations in the three base pairs farthest from the PAM when the rest of the target sites have optimal interactions with the Cas9:sgRNA. For all panels, graphs were generated from pre-selection library sequences and post-selection library sequences with an n≥5,604 and n≥158,424, respectively.

FIGS. **17**A-L. Tolerance of mutations distal to PAM in CLTA4 target sites. Distributions of mutations are shown for in vitro selection on 200 nM pre-selection library with 1000 nM Cas9:CLTA4 sgRNA v2.1. The number of mutations shown are in a 1-12 base pair target site subsequence farthest from the PAM (a-l) when the rest of the target site, including the PAM, contains only on-target base pairs. The pre-selection and post-selection distributions are similar for up to three base pairs, demonstrating tolerance for target sites with mutations in the three base pairs farthest from the PAM when the rest of the target sites have optimal interactions with the Cas9:sgRNA. For all panels, graphs were generated from pre-selection library sequences and post-selection library sequences with an n≥2,323 and n≥21,819, respectively.

FIGS. **18**A-D. Positional specificity patterns for 100 nM Cas9:sgRNA v2.1. Positional specificity, defined as the sum of the magnitude of the specificity score for each of the four possible base pairs recognized at a certain position in the target site, is plotted for each target site under enzyme-limiting conditions for sgRNA v2.1 (A-D). The positional specificity is shown as a value normalized to the maximum positional specificity value of the target site. Positional specificity is highest at the end of the target site proximal to the PAM and is lowest in the middle of the target site and in the several nucleotides most distal to the PAM.

FIGS. **19**A-D. Positional specificity patterns for 1000 nM Cas9:sgRNA v1.0. Positional specificity, defined as the sum of the magnitude of the specificity score for each of the four possible base pairs recognized at a certain position in the target site, is plotted for each target site under enzyme-excess conditions with sgRNA v1.0 (A-D). The positional specificity is shown as a value normalized to the maximum positional specificity value of the target site. Positional specificity is relatively constant across the target site but is lowest in the middle of the target site and in the several nucleotides most distal to the PAM.

FIGS. **20**A-D. Positional specificity patterns for 1000 nM Cas9:sgRNA v2.1. Positional specificity, defined as the sum of the magnitude of the specificity score for each of the four possible base pairs recognized at a certain position in the target site, is plotted for each target site under enzyme-excess conditions with sgRNA v2.1 (A-D). The positional specificity is shown as a value normalized to the maximum positional specificity value of the target site. Positional specificity is relatively constant across the target site but is lowest in the middle of the target site and in the several nucleotides most distal to the PAM.

FIGS. **21**A-D. PAM nucleotide preferences. The abundance in the pre-selection library and post-selection libraries under enzyme-limiting or enzyme-excess conditions are shown for all 16 possible PAM dinucleotides for selections with CLTA1 (a), CLTA2 (b), CLTA3 (c), and CLTA4 (d) sgRNA v2.1. GG dinucleotides increased in abundance in the post-selection libraries, while the other possible PAM dinucleotides decreased in abundance after the selection.

FIGS. **22**A-D. PAM nucleotide preferences for on-target sites. Only post-selection library members containing no mutations in the 20 base pairs specified by the guide RNAs were included in this analysis. The abundance in the pre-selection library and post-selection libraries under enzyme-limiting and enzyme-excess conditions are shown for all 16 possible PAM dinucleotides for selections with CLTA1 (A), CLTA2 (B), CLTA3 (C), and CLTA4 (D) sgRNA v2.1. GG dinucleotides increased in abundance in the post-selection libraries, while the other possible PAM dinucleotides generally decreased in abundance after the selection, although this effect for the enzyme-excess concentrations of Cas9:sgRNA was modest or non-existent for many dinucleotides.

FIGS. **23**A-D. PAM dinucleotide specificity scores. The specificity scores under enzyme-limiting and enzyme-excess conditions are shown for all 16 possible PAM dinucleotides (positions 2 and 3 of the three-nucleotide NGG PAM) for selections with CLTA1 (A), CLTA2 (B), CLTA3 (C), and CLTA4 (D) sgRNA v2.1. The specificity score indicates the enrichment of the PAM dinucleotide in the post-selection library relative to the pre-selection library, normalized to the maximum possible enrichment of that dinucleotide. A specificity score of +1.0 indicates that a dinucleotide is 100% enriched in the post-selection library, and a specificity score of −1.0 indicates that a dinucleotide is 100% de-enriched. GG dinucleotides were the most enriched in the post-selection libraries, and AG, GA, GC, GT, and TG show less relative de-enrichment compared to the other possible PAM dinucleotides.

FIGS. **24**A-D. PAM dinucleotide specificity scores for on-target sites. Only post-selection library members containing no mutations in the 20 base pairs specified by the guide RNAs were included in this analysis. The specificity scores under enzyme-limiting and enzyme-excess conditions are shown for all 16 possible PAM dinucleotides (positions 2 and 3 of the

three-nucleotide NGG PAM) for selections with CLTA1 (A), CLTA2 (B), CLTA3 (C), and CLTA4 (D) sgRNA v2.1. The specificity score indicates the enrichment of the PAM dinucleotide in the post-selection library relative to the pre-selection library, normalized to the maximum possible enrichment of that dinucleotide. A specificity score of +1.0 indicates that a dinucleotide is 100% enriched in the post-selection library, and a specificity score of −1.0 indicates that a dinucleotide is 100% de-enriched. GG dinucleotides were the most enriched in the post-selection libraries, AG and GA nucleotides were neither enriched or de-enriched in at least one selection condition, and GC, GT, and TG show less relative de-enrichment compared to the other possible PAM dinucleotides.

FIGS. **25**A-D. Effects of Cas9:sgRNA concentration on specificity. Positional specificity changes between enzyme-limiting (200 nM DNA, 100 nM Cas9:sgRNA v2.1) and enzyme-excess (200 nM DNA, 1000 nM Cas9:sgRNA v2.1) conditions are shown for selections with sgRNAs targeting CLTA1 (A), CLTA2 (B), CLTA3 (C), and CLTA4 (D) target sites. Lines indicate the maximum possible change in positional specificity for a given position. The highest changes in specificity occur proximal to the PAM as enzyme concentration is increased.

FIGS. **26**A-D. Effects of sgRNA architecture on specificity. Positional specificity changes between Cas9:sgRNA v1.0 and Cas9:sgRNA v2.1 under enzyme-excess (200 nM DNA, 1000 nM Cas9:sgRNA v2.1) conditions are shown for selections with sgRNAs targeting CLTA1 (A), CLTA2 (B), CLTA3 (C), and CLTA4 (D) target sites. Lines indicate the maximum possible change in positional specificity for a given position.

FIG. **27**. Cas9:guide RNA cleavage of off-target DNA sequences in vitro. Discrete DNA cleavage assays on a 96-bp linear substrate were performed with 200 nM DNA and 1000 nM Cas9:CLTA4 v2.1 sgRNA for the on-target CLTA4 site (CLTA4-0) and five CLTA4 off-target sites identified by in vitro selection. Enrichment values shown are from the in vitro selection with 1000 nM Cas9:CLTA4 v2.1 sgRNA. CLTA4-1 and CLTA4-3 were the most highly enriched sequences under these conditions. CLTA4-2a, CLTA4-2b, and CLTA4-2c are two-mutation sequences that represent a range of enrichment values from high enrichment to no enrichment to high de-enrichment. Lowercase letters indicate mutations relative to the on-target CLTA4 site. The enrichment values are qualitatively consistent with the observed amount of cleavage in vitro.

FIG. **28**. Effect of guide RNA architecture and Cas9:sgRNA concentration on in vitro cleavage of an off-target site. Discrete DNA cleavage assays on a 96-bp linear substrate were performed with 200 nM DNA and 100 nM Cas9:v1.0 sgRNA, 100 nM Cas9:v2.1 sgRNA, 1000 nM Cas9:v1.0 sgRNA, or 1000 nM Cas9:v2.1 sgRNA for the CLTA4-3 off-target site (5' GggGATGTAGTGTTTCCACtGGG—mutations are shown in lowercase letters). DNA cleavage is observed under all four conditions tested, and cleavage rates are higher under enzyme-excess conditions, or with v2.1 sgRNA compared with v1.0 sgRNA.

DEFINITIONS

As used herein and in the claims, the singular forms "a," "an," and "the" include the singular and the plural reference unless the context clearly indicates otherwise. Thus, for example, a reference to "an agent" includes a single agent and a plurality of such agents.

The term "Cas9" or "Cas9 nuclease" refers to an RNA-guided nuclease comprising a Cas9 protein, or a fragment thereof. A Cas9 nuclease is also referred to sometimes as a casnl nuclease or a CRISPR (clustered regularly interspaced short palindromic repeat)-associated nuclease. CRISPR is an adaptive immune system that provides protection against mobile genetic elements (e.g., viruses, transposable elements and conjugative plasmids). CRISPR clusters contain spacers, sequences complementary to antecedent mobile elements, and target invading nucleic acids. CRISPR clusters are transcribed and processed into CRISPR RNA (crRNA). In type II CRISPR systems correct processing of pre-crRNA requires a trans-encoded small RNA (tracrRNA), endogenous ribonuclease 3 (rnc) and a Cas9 protein. The tracrRNA serves as a guide for ribonuclease 3-aided processing of pre-crRNA. Subsequently, Cas9/crRNA/tracrRNA endonucleolytically cleaves linear or circular dsDNA target complementary to the spacer. The target strand not complementary to crRNA is first cut endonucleolytically, then trimmed 3'-5' exonucleolytically. In nature, DNA-binding and cleavage typically requires protein and both RNA species. However, single guide RNAs ("sgRNA", or simply "gNRA") can be engineered so as to incorporate aspects of both the crRNA and tracrRNA into a single RNA molecule. See, e.g., Jinek M., Chylinski K., Fonfara I., Hauer M., Doudna J. A., Charpentier E. *Science* 337: 816-821(2012), the entire contents of which is hereby incorporated by reference. Cas9 recognizes a short motif in the CRISPR repeat sequences (the PAM or protospacer adjacent motif) to help distinguish self versus non-self. Cas9 nuclease sequences and structures are well known to those of skill in the art (see, e.g., "Complete genome sequence of an M1 strain of *Streptococcus pyogenes*." Ferretti J. J., McShan W. M., Ajdic D. J., Savic D. J., Savic G., Lyon K., Primeaux C., Sezate S., Suvorov A. N., Kenton S., Lai H. S., Lin S. P., Qian Y., Jia H. G., Najar F. Z., Ren Q., Zhu H., Song L. expand/collapse author list McLaughlin R. E., Proc. Natl. Acad. Sci. U.S.A. 98:4658-4663(2001); "CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III." Deltcheva E., Chylinski K., Sharma C. M., Gonzales K., Chao Y., Pirzada Z. A., Eckert M. R., Vogel J., Charpentier E., Nature 471:602-607(2011); and "A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity." Jinek M., Chylinski K., Fonfara I., Hauer M., Doudna J. A., Charpentier E. *Science* 337:816-821(2012), the entire contents of each of which are incorporated herein by reference). Cas9 orthologs have been described in various species, including, but not limited to, *S. pyogenes* and *S. thermophilus*. Additional suitable Cas9 nucleases and sequences will be apparent to those of skill in the art based on this disclosure, and such Cas9 nucleases and sequences include Cas9 sequences from the organisms and loci disclosed in Chylinski, Rhun, and Charpentier, "The tracrRNA and Cas9 families of type II CRISPR-Cas immunity systems" (2013) RNA Biology 10:5, 726-737; the entire contents of which are incorporated herein by reference. In some embodiments, proteins comprising Cas9 or fragments thereof proteins are referred to as "Cas9 variants." A Cas9 variant shares homology to Cas9, or a fragment thereof. For example a Cas9 variant is at least about 70% identical, at least about 80% identical, at least about 90% identical, at least about 95% identical, at least about 98% identical, at least about 99% identical, at least about 99.5% identical, or at least about 99.9% to wild type Cas9. In some embodiments, the Cas9 variant comprises a fragment of Cas9 (e.g., a gRNA binding domain or a DNA-cleavage domain), such that the fragment is at least about 70% identical, at least about 80% identical, at least about 90% identical, at least about 95% identical, at least about 98% identical, at least about 99% identical, at least about 99.5% identical, or at least about 99.9% to the corresponding frag-

ment of wild type Cas9. In some embodiments, wild type Cas9 corresponds to Cas9 from *Streptococcus pyogenes* (NCBI Reference Sequence: NC_017053.1, SEQ ID NO:40 (nucleotide); SEQ ID NO:41 (amino acid)).

(SEQ ID NO: 40)

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CTGA
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(SEQ ID NO: 41)

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NFKSNFDLAEDAKLQLSKDTYDDDLDNLLAQIGDQYADLFLAAKNLSDAI
LLSDILRVNSEITKAPLSASMIKRYDEHHQDLTLLKALVRQQLPEKYKEI
FFDQSKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNREDLLR
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The term "concatemer," as used herein in the context of nucleic acid molecules, refers to a nucleic acid molecule that contains multiple copies of the same DNA sequences linked in a series. For example, a concatemer comprising ten copies of a specific sequence of nucleotides (e.g., $[XYZ]_{10}$), would comprise ten copies of the same specific sequence linked to each other in series, e.g., 5'-XYZXYZXYZXYZXYZX-YZXYZXYZXYZXYZ-3'. A concatemer may comprise any number of copies of the repeat unit or sequence, e.g., at least 2 copies, at least 3 copies, at least 4 copies, at least 5 copies, at least 10 copies, at least 100 copies, at least 1000 copies, etc. An example of a concatemer of a nucleic acid sequence comprising a nuclease target site and a constant insert sequence would be $[(\text{target site})\text{-}(\text{constant insert sequence})]_{300}$. A concatemer may be a linear nucleic acid molecule, or may be circular.

The terms "conjugating," "conjugated," and "conjugation" refer to an association of two entities, for example, of two molecules such as two proteins, two domains (e.g., a binding domain and a cleavage domain), or a protein and an agent, e.g., a protein binding domain and a small molecule. In some aspects, the association is between a protein (e.g., RNA-programmable nuclease) and a nucleic acid (e.g., a guide RNA). The association can be, for example, via a direct or indirect (e.g., via a linker) covalent linkage or via non-covalent interactions. In some embodiments, the association is covalent. In some embodiments, two molecules are conjugated via a linker connecting both molecules. For example, in some embodiments where two proteins are conjugated to each other, e.g., a binding domain and a cleavage domain of an engineered nuclease, to form a protein fusion, the two proteins may be conjugated via a polypeptide linker, e.g., an amino acid sequence connecting the C-terminus of one protein to the N-terminus of the other protein.

The term "consensus sequence," as used herein in the context of nucleic acid sequences, refers to a calculated sequence representing the most frequent nucleotide residues found at each position in a plurality of similar sequences. Typically, a consensus sequence is determined by sequence alignment in which similar sequences are compared to each other and similar sequence motifs are calculated. In the context of nuclease target site sequences, a consensus sequence of a nuclease target site may, in some embodiments, be the sequence most frequently bound, or bound with the highest affinity, by a given nuclease. With respect to RNA-programmable nuclease (e.g., Cas9) target site sequences, the consensus sequence may, in some embodiments, be the sequence or region to which a gRNA, or a plurality of gRNAs, is expected or designed to bind, e.g., based on complementary base pairing.

The term "effective amount," as used herein, refers to an amount of a biologically active agent that is sufficient to elicit a desired biological response. For example, in some embodiments, an effective amount of a nuclease may refer to the amount of the nuclease that is sufficient to induce cleavage of a target site specifically bound and cleaved by the nuclease. As will be appreciated by the skilled artisan, the effective amount of an agent, e.g., a nuclease, a hybrid protein, or a polynucleotide, may vary depending on various factors as, for example, on the desired biological response, the specific allele, genome, target site, cell, or tissue being targeted, and the agent being used.

The term "enediyne," as used herein, refers to a class of bacterial natural products characterized by either nine- and ten-membered rings containing two triple bonds separated by a double bond (see, e.g., K. C. Nicolaou; A. L. Smith; E. W. Yue (1993). "Chemistry and biology of natural and designed enediynes". PNAS 90 (13): 5881-5888; the entire contents of which are incorporated herein by reference). Some enediynes are capable of undergoing Bergman cyclization, and the resulting diradical, a 1,4-dehydrobenzene derivative, is capable of abstracting hydrogen atoms from the sugar backbone of DNA which results in DNA strand cleavage (see, e.g., S. Walker; R. Landovitz; W. D. Ding; G. A. Ellestad; D. Kahne (1992). "Cleavage behavior of calicheamicin gamma 1 and calicheamicin T". Proc Natl Acad Sci U.S.A. 89 (10): 4608-12; the entire contents of which are incorporated herein by reference). Their reactivity with DNA confers an antibiotic character to many enediynes, and some enediynes are clinically investigated as anticancer antibiotics. Nonlimiting examples of enediynes are dynemicin, neocarzinostatin, calicheamicin, esperamicin (see, e.g., Adrian L. Smith and K. C. Bicolaou, "The Enediyne Antibiotics" J. Med. Chem., 1996, 39 (11), pp 2103-2117; and Donald Borders, "Enediyne antibiotics as antitumor agents," Informa Healthcare; 1st edition (Nov. 23, 1994, ISBN-10: 0824789385; the entire contents of which are incorporated herein by reference).

The term "homing endonuclease," as used herein, refers to a type of restriction enzymes typically encoded by introns or inteins Edgell DR (February 2009). "Selfish DNA: homing endonucleases find a home". *Curr Biol* 19 (3): R115-R117; Jasin M (June 1996). "Genetic manipulation of genomes with rare-cutting endonucleases". *Trends Genet* 12 (6): 224-8; Burt A, Koufopanou V (December 2004). "Homing endonuclease genes: the rise and fall and rise again of a selfish element". *Curr Opin Genet Dev* 14 (6): 609-15; the entire contents of which are incorporated herein by reference. Homing endonuclease recognition sequences are long enough to occur randomly only with a very low probability (approximately once every 7×10^{10} bp), and are normally found in only one instance per genome.

The term "library," as used herein in the context of nucleic acids or proteins, refers to a population of two or more different nucleic acids or proteins, respectively. For example, a library of nuclease target sites comprises at least two nucleic acid molecules comprising different nuclease target sites. In some embodiments, a library comprises at least 10^1, at least 10^2, at least 10^3, at least 10^4, at least 10^5, at least 10^6, at least 10^7, at least 10^8, at least 10^9, at least 10^{10}, at least 10^{11}, at least 10^{12}, at least 10^{13}, at least 10^{14}, or at least 10^{15} different nucleic acids or proteins. In some embodiments, the members of the library may comprise randomized sequences, for example, fully or partially randomized sequences. In some embodiments, the library comprises nucleic acid molecules that are unrelated to each other, e.g., nucleic acids comprising fully randomized sequences. In other embodiments, at least some members of the library may be related, for example, they may be variants or derivatives of a particular sequence, such as a consensus target site sequence.

The term "linker," as used herein, refers to a chemical group or a molecule linking two adjacent molecules or moieties, e.g., a binding domain and a cleavage domain of a nuclease. Typically, the linker is positioned between, or flanked by, two groups, molecules, or other moieties and connected to each one via a covalent bond, thus connecting the two. In some embodiments, the linker is an amino acid or a plurality of amino acids (e.g., a peptide or protein). In some embodiments, the linker is an organic molecule, group, polymer, or chemical moiety.

The term "nuclease," as used herein, refers to an agent, for example a protein or a small molecule, capable of cleaving a phosphodiester bond connecting nucleotide residues in a nucleic acid molecule. In some embodiments, a nuclease is a protein, e.g., an enzyme that can bind a nucleic acid molecule and cleave a phosphodiester bond connecting nucleotide residues within the nucleic acid molecule. A nuclease may be an endonuclease, cleaving a phosphodiester bonds within a polynucleotide chain, or an exonuclease, cleaving a phosphodiester bond at the end of the polynucleotide chain. In some embodiments, a nuclease is a site-specific nuclease, binding and/or cleaving a specific phosphodiester bond within a specific nucleotide sequence, which is also referred to herein as the "recognition sequence," the "nuclease target site," or the "target site." In some embodiments, a nuclease is a RNA-guided (i.e., RNA-programmable) nuclease, which complexes with (e.g., binds with) an RNA having a sequence that complements a target site, thereby providing the sequence specificity of the nuclease. In some embodiments, a nuclease recognizes a single stranded target site, while in other embodiments, a nuclease recognizes a double-stranded target site, for example a double-stranded DNA target site. The target sites of many naturally occurring nucleases, for example, many naturally occurring DNA restriction nucleases, are well known to those of skill in the art. In many cases, a DNA nuclease, such as EcoRI, HindIII, or BamHI, recognize a palindromic, double-stranded DNA target site of 4 to 10 base pairs in length, and cut each of the two DNA strands at a specific position within the target site. Some endonucleases cut a double-stranded nucleic acid target site symmetrically, i.e., cutting both strands at the same position so that the ends comprise base-paired nucleotides, also referred to herein as blunt ends. Other endonucleases cut a double-stranded nucleic acid target sites asymmetrically, i.e., cutting each strand at a different position so that the ends comprise unpaired nucleotides. Unpaired nucleotides at the end of a double-stranded DNA molecule are also referred to as "overhangs," e.g., as "5'-overhang" or as "3'-overhang," depending on whether the unpaired nucleotide(s) form(s) the

5' or the 3' end of the respective DNA strand. Double-stranded DNA molecule ends ending with unpaired nucleotide(s) are also referred to as sticky ends, as they can "stick to" other double-stranded DNA molecule ends comprising complementary unpaired nucleotide(s). A nuclease protein typically comprises a "binding domain" that mediates the interaction of the protein with the nucleic acid substrate, and also, in some cases, specifically binds to a target site, and a "cleavage domain" that catalyzes the cleavage of the phosphodiester bond within the nucleic acid backbone. In some embodiments a nuclease protein can bind and cleave a nucleic acid molecule in a monomeric form, while, in other embodiments, a nuclease protein has to dimerize or multimerize in order to cleave a target nucleic acid molecule. Binding domains and cleavage domains of naturally occurring nucleases, as well as modular binding domains and cleavage domains that can be fused to create nucleases binding specific target sites, are well known to those of skill in the art. For example, zinc fingers or transcriptional activator like elements can be used as binding domains to specifically bind a desired target site, and fused or conjugated to a cleavage domain, for example, the cleavage domain of FokI, to create an engineered nuclease cleaving the target site.

The terms "nucleic acid" and "nucleic acid molecule," as used herein, refer to a compound comprising a nucleobase and an acidic moiety, e.g., a nucleoside, a nucleotide, or a polymer of nucleotides. Typically, polymeric nucleic acids, e.g., nucleic acid molecules comprising three or more nucleotides are linear molecules, in which adjacent nucleotides are linked to each other via a phosphodiester linkage. In some embodiments, "nucleic acid" refers to individual nucleic acid residues (e.g. nucleotides and/or nucleosides). In some embodiments, "nucleic acid" refers to an oligonucleotide chain comprising three or more individual nucleotide residues. As used herein, the terms "oligonucleotide" and "polynucleotide" can be used interchangeably to refer to a polymer of nucleotides (e.g., a string of at least three nucleotides). In some embodiments, "nucleic acid" encompasses RNA as well as single and/or double-stranded DNA. Nucleic acids may be naturally occurring, for example, in the context of a genome, a transcript, an mRNA, tRNA, rRNA, siRNA, snRNA, a plasmid, cosmid, chromosome, chromatid, or other naturally occurring nucleic acid molecule. On the other hand, a nucleic acid molecule may be a non-naturally occurring molecule, e.g., a recombinant DNA or RNA, an artificial chromosome, an engineered genome, or fragment thereof, or a synthetic DNA, RNA, DNA/RNA hybrid, or including non-naturally occurring nucleotides or nucleosides. Furthermore, the terms "nucleic acid," "DNA," "RNA," and/or similar terms include nucleic acid analogs, i.e. analogs having other than a phosphodiester backbone. Nucleic acids can be purified from natural sources, produced using recombinant expression systems and optionally purified, chemically synthesized, etc. Where appropriate, e.g., in the case of chemically synthesized molecules, nucleic acids can comprise nucleoside analogs such as analogs having chemically modified bases or sugars, and backbone modifications. A nucleic acid sequence is presented in the 5' to 3' direction unless otherwise indicated. In some embodiments, a nucleic acid is or comprises natural nucleosides (e.g. adenosine, thymidine, guanosine, cytidine, uridine, deoxyadenosine, deoxythymidine, deoxyguanosine, and deoxycytidine); nucleoside analogs (e.g., 2-aminoadenosine, 2-thiothymidine, inosine, pyrrolo-pyrimidine, 3-methyl adenosine, 5-methylcytidine, 2-aminoadenosine, C5-bromouridine, C5-fluorouridine, C5-iodouridine, C5-propynyl-uridine, C5-propynyl-cytidine, C5-methylcytidine, 2-aminoadenosine, 7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine, O(6)-methylguanine, and 2-thiocytidine); chemically modified bases; biologically modified bases (e.g., methylated bases); intercalated bases; modified sugars (e.g., 2'-fluororibose, ribose, 2'-deoxyribose, arabinose, and hexose); and/or modified phosphate groups (e.g., phosphorothioates and 5'-N-phosphoramidite linkages).

The term "pharmaceutical composition," as used herein, refers to a composition that can be administrated to a subject in the context of treatment of a disease or disorder. In some embodiments, a pharmaceutical composition comprises an active ingredient, e.g., a nuclease or a nucleic acid encoding a nuclease, and a pharmaceutically acceptable excipient.

The term "proliferative disease," as used herein, refers to any disease in which cell or tissue homeostasis is disturbed in that a cell or cell population exhibits an abnormally elevated proliferation rate. Proliferative diseases include hyperproliferative diseases, such as pre-neoplastic hyperplastic conditions and neoplastic diseases. Neoplastic diseases are characterized by an abnormal proliferation of cells and include both benign and malignant neoplasias. Malignant neoplasia is also referred to as cancer.

The terms "protein," "peptide," and "polypeptide" are used interchangeably herein, and refer to a polymer of amino acid residues linked together by peptide (amide) bonds. The terms refer to a protein, peptide, or polypeptide of any size, structure, or function. Typically, a protein, peptide, or polypeptide will be at least three amino acids long. A protein, peptide, or polypeptide may refer to an individual protein or a collection of proteins. One or more of the amino acids in a protein, peptide, or polypeptide may be modified, for example, by the addition of a chemical entity such as a carbohydrate group, a hydroxyl group, a phosphate group, a farnesyl group, an isofarnesyl group, a fatty acid group, a linker for conjugation, functionalization, or other modification, etc. A protein, peptide, or polypeptide may also be a single molecule or may be a multi-molecular complex. A protein, peptide, or polypeptide may be just a fragment of a naturally occurring protein or peptide. A protein, peptide, or polypeptide may be naturally occurring, recombinant, or synthetic, or any combination thereof. A protein may comprise different domains, for example, a nucleic acid binding domain and a nucleic acid cleavage domain. In some embodiments, a protein comprises a proteinaceous part, e.g., an amino acid sequence constituting a nucleic acid binding domain, and an organic compound, e.g., a compound that can act as a nucleic acid cleavage agent. In some embodiments, a protein is in a complex with, or is in association with, a nucleic acid, e.g., RNA.

The term "randomized," as used herein in the context of nucleic acid sequences, refers to a sequence or residue within a sequence that has been synthesized to incorporate a mixture of free nucleotides, for example, a mixture of all four nucleotides A, T, G, and C. Randomized residues are typically represented by the letter N within a nucleotide sequence. In some embodiments, a randomized sequence or residue is fully randomized, in which case the randomized residues are synthesized by adding equal amounts of the nucleotides to be incorporated (e.g., 25% T, 25% A, 25% G, and 25% C) during the synthesis step of the respective sequence residue. In some embodiments, a randomized sequence or residue is partially randomized, in which case the randomized residues are synthesized by adding non-equal amounts of the nucleotides to be incorporated (e.g., 79% T, 7% A, 7% G, and 7% C) during the synthesis step of the respective sequence residue. Partial randomization allows for the generation of sequences that are templated on a given sequence, but have incorporated mutations at a desired frequency. For example, if a known nuclease

target site is used as a synthesis template, partial randomization in which at each step the nucleotide represented at the respective residue is added to the synthesis at 79%, and the other three nucleotides are added at 7% each, will result in a mixture of partially randomized target sites being synthesized, which still represent the consensus sequence of the original target site, but which differ from the original target site at each residue with a statistical frequency of 21% for each residue so synthesized (distributed binomially). In some embodiments, a partially randomized sequence differs from the consensus sequence by more than 5%, more than 10%, more than 15%, more than 20%, more than 25%, or more than 30% on average, distributed binomially. In some embodiments, a partially randomized sequence differs from the consensus site by no more than 10%, no more than 15%, no more than 20%, no more than 25%, nor more than 30%, no more than 40%, or no more than 50% on average, distributed binomially.

The term "RNA-programmable nuclease," and "RNA-guided nuclease" are used interchangeably herein and refer to a nuclease that forms a complex with (e.g., binds or associates with) one or more RNA that is not a target for cleavage. In some embodiments, an RNA-programmable nuclease, when in a complex with an RNA, may be referred to as a nuclease: RNA complex. Typically, the bound RNA(s) is referred to as a guide RNA (gRNA) or a single-guide RNA (sgRNA). The gRNA/sgRNA comprises a nucleotide sequence that complements a target site, which mediates binding of the nuclease/RNA complex to said target site and providing the sequence specificity of the nuclease:RNA complex. In some embodiments, the RNA-programmable nuclease is the (CRISPR-associated system) Cas9 endonuclease, for example Cas9 (Csn1) from *Streptococcus pyogenes* (see, e.g., "Complete genome sequence of an M1 strain of *Streptococcus pyogenes*." Ferretti J. J., McShan W. M., Ajdic D. J., Savic D. J., Savic G., Lyon K., Primeaux C., Sezate S., Suvorov A. N., Kenton S., Lai H. S., Lin S. P., Qian Y., Jia H. G., Najar F. Z., Ren Q., Zhu H., Song L. expand/collapse author list McLaughlin R. E., Proc. Natl. Acad. Sci. U.S.A. 98:4658-4663(2001); "CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III." Deltcheva E., Chylinski K., Sharma C. M., Gonzales K., Chao Y., Pirzada Z. A., Eckert M. R., Vogel J., Charpentier E., Nature 471:602-607 (2011); and "A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity." Jinek M., Chylinski K., Fonfara I., Hauer M., Doudna J. A., Charpentier E. Science 337:816-821(2012), the entire contents of each of which are incorporated herein by reference

Because RNA-programmable nucleases (e.g., Cas9) use RNA:DNA hybridization to determine target DNA cleavage sites, these proteins are able to cleave, in principle, any sequence specified by the guide RNA. Methods of using RNA-programmable nucleases, such as Cas9, for site-specific cleavage (e.g., to modify a genome) are known in the art (See e.g., Cong, L. et al. Multiplex genome engineering using CRISPR/Cas systems. *Science* 339, 819-823 (2013); Mali, P. et al. RNA-guided human genome engineering via Cas9. *Science* 339, 823-826 (2013); Hwang, W. Y. et al. Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nature biotechnology* 31, 227-229 (2013); Jinek, M. et al. RNA-programmed genome editing in human cells. eLife 2, e00471 (2013); Dicarlo, J. E. et al. Genome engineering in *Saccharomyces cerevisiae* using CRISPR-Cas systems. *Nucleic acids research* (2013); Jiang, W. et al. RNA-guided editing of bacterial genomes using CRISPR-Cas systems. *Nature biotechnology* 31, 233-239 (2013); the entire contents of each of which are incorporated herein by reference).

The terms "small molecule" and "organic compound" are used interchangeably herein and refer to molecules, whether naturally-occurring or artificially created (e.g., via chemical synthesis) that have a relatively low molecular weight. Typically, an organic compound contains carbon. An organic compound may contain multiple carbon-carbon bonds, stereocenters, and other functional groups (e.g., amines, hydroxyl, carbonyls, or heterocyclic rings). In some embodiments, organic compounds are monomeric and have a molecular weight of less than about 1500 g/mol. In certain embodiments, the molecular weight of the small molecule is less than about 1000 g/mol or less than about 500 g/mol. In certain embodiments, the small molecule is a drug, for example, a drug that has already been deemed safe and effective for use in humans or animals by the appropriate governmental agency or regulatory body. In certain embodiments, the organic molecule is known to bind and/or cleave a nucleic acid. In some embodiments, the organic compound is an enediyne. In some embodiments, the organic compound is an antibiotic drug, for example, an anticancer antibiotic such as dynemicin, neocarzinostatin, calicheamicin, esperamicin, bleomycin, or a derivative thereof.

The term "subject," as used herein, refers to an individual organism, for example, an individual mammal. In some embodiments, the subject is a human. In some embodiments, the subject is a non-human mammal. In some embodiments, the subject is a non-human primate. In some embodiments, the subject is a rodent. In some embodiments, the subject is a sheep, a goat, a cattle, a cat, or a dog. In some embodiments, the subject is a vertebrate, an amphibian, a reptile, a fish, an insect, a fly, or a nematode.

The terms "target nucleic acid," and "target genome," as used herein in the context of nucleases, refer to a nucleic acid molecule or a genome, respectively, that comprises at least one target site of a given nuclease.

The term "target site," used herein interchangeably with the term "nuclease target site," refers to a sequence within a nucleic acid molecule that is bound and cleaved by a nuclease. A target site may be single-stranded or double-stranded. In the context of nucleases that dimerize, for example, nucleases comprising a FokI DNA cleavage domain, a target sites typically comprises a left-half site (bound by one monomer of the nuclease), a right-half site (bound by the second monomer of the nuclease), and a spacer sequence between the half sites in which the cut is made. This structure ([left-half site]-[spacer sequence]-[right-half site]) is referred to herein as an LSR structure. In some embodiments, the left-half site and/or the right-half site is between 10-18 nucleotides long. In some embodiments, either or both half-sites are shorter or longer. In some embodiments, the left and right half sites comprise different nucleic acid sequences. In the context of zinc finger nucleases, target sites may, in some embodiments comprise two half-sites that are each 6-18 bp long flanking a non-specified spacer region that is 4-8 bp long. In the context of TALENs, target sites may, in some embodiments, comprise two half-sites sites that are each 10-23 bp long flanking a non-specified spacer region that is 10-30 bp long. In the context of RNA-guided (e.g., RNA-programmable) nucleases, a target site typically comprises a nucleotide sequence that is complementary to the sgRNA of the RNA-programmable nuclease, and a protospacer adjacent motif (PAM) at the 3' end adjacent to the sgRNA-complementary sequence. For the RNA-guided nuclease Cas9, the target site may be, in some embodiments, 20 base pairs plus a 3 base pair PAM (e.g., NNN, wherein N represents any nucleotide). Typically, the first nucleotide of a PAM can be any nucleotide, while the two downstream nucleotides are specified depend-

ing on the specific RNA-guided nuclease. Exemplary target sites for RNA-guided nucleases, such as Cas9, are known to those of skill in the art and include, without limitation, NNG, NGN, NAG, and NGG, wherein N represents any nucleotide. In addition, Cas9 nucleases from different species (e.g., *S. thermophilus* instead of *S. pyogenes*) recognizes a PAM that comprises the sequence NGGNG. Additional PAM sequences are known, including, but not limited to NNAGAAW and NAAR (see, e.g., Esvelt and Wang, Molecular Systems Biology, 9:641 (2013), the entire contents of which are incorporated herein by reference). For example, the target site of an RNA-guided nuclease, such as, e.g., Cas9, may comprise the structure $[N_Z]$-[PAM], where each N is, independently, any nucleotide, and $_Z$ is an integer between 1 and 50. In some embodiments, $_Z$ is at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 25, at least 30, at least 35, at least 40, at least 45, or at least 50. In some embodiments, $_Z$ is 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50. In some embodiments, Z is 20.

The term "Transcriptional Activator-Like Effector," (TALE) as used herein, refers to bacterial proteins comprising a DNA binding domain, which contains a highly conserved 33-34 amino acid sequence comprising a highly variable two-amino acid motif (Repeat Variable Diresidue, RVD). The RVD motif determines binding specificity to a nucleic acid sequence, and can be engineered according to methods well known to those of skill in the art to specifically bind a desired DNA sequence (see, e.g., Miller, Jeffrey; et. al. (February 2011). "A TALE nuclease architecture for efficient genome editing". *Nature Biotechnology* 29 (2): 143-8; Zhang, Feng; et. al. (February 2011). "Efficient construction of sequence-specific TAL effectors for modulating mammalian transcription". *Nature Biotechnology* 29 (2): 149-53; Geiβler, R.; Scholze, H.; Hahn, S.; Streubel, J.; Bonas, U.; Behrens, S. E.; Boch, J. (2011), Shiu, Shin-Han. ed. "Transcriptional Activators of Human Genes with Programmable DNA-Specificity". *PLoS ONE* 6 (5): e19509; Boch, Jens (February 2011). "TALEs of genome targeting". *Nature Biotechnology* 29 (2): 135-6; Boch, Jens; et. al. (December 2009). "Breaking the Code of DNA Binding Specificity of TAL-Type III Effectors". *Science* 326 (5959): 1509-12; and Moscou, Matthew J.; Adam J. Bogdanove (December 2009). "A Simple Cipher Governs DNA Recognition by TAL Effectors". *Science* 326 (5959): 1501; the entire contents of each of which are incorporated herein by reference). The simple relationship between amino acid sequence and DNA recognition has allowed for the engineering of specific DNA binding domains by selecting a combination of repeat segments containing the appropriate RVDs.

The term "Transcriptional Activator-Like Element Nuclease," (TALEN) as used herein, refers to an artificial nuclease comprising a transcriptional activator like effector DNA binding domain to a DNA cleavage domain, for example, a FokI domain. A number of modular assembly schemes for generating engineered TALE constructs have been reported (see e.g., Zhang, Feng; et. al. (February 2011). "Efficient construction of sequence-specific TAL effectors for modulating mammalian transcription". *Nature Biotechnology* 29 (2): 149-53; Geiβler, R.; Scholze, H.; Hahn, S.; Streubel, J.; Bonas, U.; Behrens, S. E.; Boch, J. (2011), Shiu, Shin-Han. ed. "Transcriptional Activators of Human Genes with Programmable DNA-Specificity". *PLoS ONE* 6 (5): e19509; Cermak, T.; Doyle, E. L.; Christian, M.; Wang, L.; Zhang, Y.; Schmidt, C.; Baller, J. A.; Somia, N. V. et al. (2011). "Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting". *Nucleic Acids Research*; Morbitzer, R.; Elsaesser, J.; Hausner, J.; Lahaye, T. (2011). "Assembly of custom TALE-type DNA binding domains by modular cloning". *Nucleic Acids Research*; Li, T.; Huang, S.; Zhao, X.; Wright, D. A.; Carpenter, S.; Spalding, M. H.; Weeks, D. P.; Yang, B. (2011). "Modularly assembled designer TAL effector nucleases for targeted gene knockout and gene replacement in eukaryotes". *Nucleic Acids Research*.; Weber, E.; Gruetzner, R.; Werner, S.; Engler, C.; Marillonnet, S. (2011). Bendahmane, Mohammed. ed. "Assembly of Designer TAL Effectors by Golden Gate Cloning". *PLoS ONE* 6 (5): e19722; the entire contents of each of which are incorporated herein by reference).

The terms "treatment," "treat," and "treating," refer to a clinical intervention aimed to reverse, alleviate, delay the onset of, or inhibit the progress of a disease or disorder, or one or more symptoms thereof, as described herein. As used herein, the terms "treatment," "treat," and "treating" refer to a clinical intervention aimed to reverse, alleviate, delay the onset of, or inhibit the progress of a disease or disorder, or one or more symptoms thereof, as described herein. In some embodiments, treatment may be administered after one or more symptoms have developed and/or after a disease has been diagnosed. In other embodiments, treatment may be administered in the absence of symptoms, e.g., to prevent or delay onset of a symptom or inhibit onset or progression of a disease. For example, treatment may be administered to a susceptible individual prior to the onset of symptoms (e.g., in light of a history of symptoms and/or in light of genetic or other susceptibility factors). Treatment may also be continued after symptoms have resolved, for example to prevent or delay their recurrence.

The term "zinc finger," as used herein, refers to a small nucleic acid-binding protein structural motif characterized by a fold and the coordination of one or more zinc ions that stabilize the fold. Zinc fingers encompass a wide variety of differing protein structures (see, e.g., Klug A, Rhodes D (1987). "Zinc fingers: a novel protein fold for nucleic acid recognition". Cold Spring Harb. Symp. Quant. Biol. 52: 473-82, the entire contents of which are incorporated herein by reference). Zinc fingers can be designed to bind a specific sequence of nucleotides, and zinc finger arrays comprising fusions of a series of zinc fingers, can be designed to bind virtually any desired target sequence. Such zinc finger arrays can form a binding domain of a protein, for example, of a nuclease, e.g., if conjugated to a nucleic acid cleavage domain. Different type of zinc finger motifs are known to those of skill in the art, including, but not limited to, Cys_2His_2, Gag knuckle, Treble clef, Zinc ribbon, Zn_2/Cys_6, and TAZ2 domain-like motifs (see, e.g., Krishna S S, Majumdar I, Grishin N V (January 2003). "Structural classification of zinc fingers: survey and summary". *Nucleic Acids Res.* 31 (2): 532-50). Typically, a single zinc finger motif binds 3 or 4 nucleotides of a nucleic acid molecule. Accordingly, a zinc finger domain comprising 2 zinc finger motifs may bind 6-8 nucleotides, a zinc finger domain comprising 3 zinc finger motifs may bind 9-12 nucleotides, a zinc finger domain comprising 4 zinc finger motifs may bind 12-16 nucleotides, and so forth. Any suitable protein engineering technique can be employed to alter the DNA-binding specificity of zinc fingers and/or design novel zinc finger fusions to bind virtually any desired target sequence from 3-30 nucleotides in length (see, e.g., Pabo C O, Peisach E, Grant R A (2001). "Design and selection of novel cys2His2 Zinc finger proteins". *Annual Review of Biochemistry* 70: 313-340; Jamieson A C, Miller J

C, Pabo C O (2003). "Drug discovery with engineered zinc-finger proteins". *Nature Reviews Drug Discovery* 2 (5): 361-368; and Liu Q, Segal D J, Ghiara J B, Barbas C F (May 1997). "Design of polydactyl zinc-finger proteins for unique addressing within complex genomes". *Proc. Natl. Acad. Sci. U.S.A.* 94 (11); the entire contents of each of which are incorporated herein by reference). Fusions between engineered zinc finger arrays and protein domains that cleave a nucleic acid can be used to generate a "zinc finger nuclease." A zinc finger nuclease typically comprises a zinc finger domain that binds a specific target site within a nucleic acid molecule, and a nucleic acid cleavage domain that cuts the nucleic acid molecule within or in proximity to the target site bound by the binding domain. Typical engineered zinc finger nucleases comprise a binding domain having between 3 and 6 individual zinc finger motifs and binding target sites ranging from 9 base pairs to 18 base pairs in length. Longer target sites are particularly attractive in situations where it is desired to bind and cleave a target site that is unique in a given genome.

The term "zinc finger nuclease," as used herein, refers to a nuclease comprising a nucleic acid cleavage domain conjugated to a binding domain that comprises a zinc finger array. In some embodiments, the cleavage domain is the cleavage domain of the type II restriction endonuclease FokI. Zinc finger nucleases can be designed to target virtually any desired sequence in a given nucleic acid molecule for cleavage, and the possibility to the design zinc finger binding domains to bind unique sites in the context of complex genomes allows for targeted cleavage of a single genomic site in living cells, for example, to achieve a targeted genomic alteration of therapeutic value. Targeting a double-strand break to a desired genomic locus can be used to introduce frame-shift mutations into the coding sequence of a gene due to the error-prone nature of the non-homologous DNA repair pathway. Zinc finger nucleases can be generated to target a site of interest by methods well known to those of skill in the art. For example, zinc finger binding domains with a desired specificity can be designed by combining individual zinc finger motifs of known specificity. The structure of the zinc finger protein Zif268 bound to DNA has informed much of the work in this field and the concept of obtaining zinc fingers for each of the 64 possible base pair triplets and then mixing and matching these modular zinc fingers to design proteins with any desired sequence specificity has been described (Pavletich N P, Pabo C O (May 1991). "Zinc finger-DNA recognition: crystal structure of a Zif268-DNA complex at 2.1 A". *Science* 252 (5007): 809-17, the entire contents of which are incorporated herein). In some embodiments, separate zinc fingers that each recognize a 3 base pair DNA sequence are combined to generate 3-, 4-, 5-, or 6-finger arrays that recognize target sites ranging from 9 base pairs to 18 base pairs in length. In some embodiments, longer arrays are contemplated. In other embodiments, 2-finger modules recognizing 6-8 nucleotides are combined to generate 4-, 6-, or 8-zinc finger arrays. In some embodiments, bacterial or phage display is employed to develop a zinc finger domain that recognizes a desired nucleic acid sequence, for example, a desired nuclease target site of 3-30 bp in length. Zinc finger nucleases, in some embodiments, comprise a zinc finger binding domain and a cleavage domain fused or otherwise conjugated to each other via a linker, for example, a polypeptide linker. The length of the linker determines the distance of the cut from the nucleic acid sequence bound by the zinc finger domain. If a shorter linker is used, the cleavage domain will cut the nucleic acid closer to the bound nucleic acid sequence, while a longer linker will result in a greater distance between the cut and the bound nucleic acid sequence. In some embodiments, the cleavage domain of a zinc finger nuclease has to dimerize in order to cut a bound nucleic acid. In some such embodiments, the dimer is a heterodimer of two monomers, each of which comprise a different zinc finger binding domain. For example, in some embodiments, the dimer may comprise one monomer comprising zinc finger domain A conjugated to a FokI cleavage domain, and one monomer comprising zinc finger domain B conjugated to a FokI cleavage domain. In this nonlimiting example, zinc finger domain A binds a nucleic acid sequence on one side of the target site, zinc finger domain B binds a nucleic acid sequence on the other side of the target site, and the dimerize FokI domain cuts the nucleic acid in between the zinc finger domain binding sites.

DETAILED DESCRIPTION OF CERTAIN EMBODIMENTS OF THE INVENTION

Introduction

Site-specific nucleases are powerful tools for targeted genome modification in vitro or in vivo. Some site specific nucleases can theoretically achieve a level of specificity for a target cleavage site that would allow one to target a single unique site in a genome for cleavage without affecting any other genomic site. It has been reported that nuclease cleavage in living cells triggers a DNA repair mechanism that frequently results in a modification of the cleaved, repaired genomic sequence, for example, via homologous recombination. Accordingly, the targeted cleavage of a specific unique sequence within a genome opens up new avenues for gene targeting and gene modification in living cells, including cells that are hard to manipulate with conventional gene targeting methods, such as many human somatic or embryonic stem cells. Nuclease-mediated modification of disease-related sequences, e.g., the CCR-5 allele in HIV/AIDS patients, or of genes necessary for tumor neovascularization, can be used in the clinical context, and two site specific nucleases are currently in clinical trials.

One important aspect in the field of site-specific nuclease-mediated modification are off-target nuclease effects, e.g., the cleavage of genomic sequences that differ from the intended target sequence by one or more nucleotides. Undesired side effects of off-target cleavage range from insertion into unwanted loci during a gene targeting event to severe complications in a clinical scenario. Off-target cleavage of sequences encoding essential gene functions or tumor suppressor genes by an endonuclease administered to a subject may result in disease or even death of the subject. Accordingly, it is desirable to characterize the cleavage preferences of a nuclease before using it in the laboratory or the clinic in order to determine its efficacy and safety. Further, the characterization of nuclease cleavage properties allows for the selection of the nuclease best suited for a specific task from a group of candidate nucleases, or for the selection of evolution products obtained from a plurality of nucleases. Such a characterization of nuclease cleavage properties may also inform the de-novo design of nucleases with enhanced properties, such as enhanced specificity or efficiency.

In many scenarios where a nuclease is employed for the targeted manipulation of a nucleic acid, cleavage specificity is a crucial feature. The imperfect specificity of some engineered nuclease binding domains can lead to off-target cleavage and undesired effects both in vitro and in vivo. Current methods of evaluating site-specific nuclease specificity, including ELISA assays, microarrays, one-hybrid systems, SELEX, and its variants, and Rosetta-based computational

predictions, are all premised on the assumption that the binding specificity of the nuclease is equivalent or proportionate to their cleavage specificity.

It was previously discovered that the prediction of nuclease off-target binding effects constitute an imperfect approximation of a nuclease's off-target cleavage effects that may result in undesired biological effects (see PCT Application WO 2013/066438; and Pattanayak, V., Ramirez, C. L., Joung, J. K. & Liu, D. R. Revealing off-target cleavage specificities of zinc-finger nucleases by in vitro selection. *Nature methods* 8, 765-770 (2011), the entire contents of each of which are incorporated herein by reference). This finding was consistent with the notion that the reported toxicity of some site specific DNA nucleases results from off-target DNA cleavage, rather than off-target binding alone.

The methods and reagents of the present disclosure represent, in some aspects, an improvement over previous methods and allow for an accurate evaluation of a given nuclease's target site specificity and provide strategies for the selection of suitable unique target sites and the design or selection of highly specific nucleases for the targeted cleavage of a single site in the context of a complex genome. For example, some previously reported methods for determining nuclease target site specificity profiles by screening libraries of nucleic acid molecules comprising candidate target sites relied on a "two-cut" in vitro selection method which requires indirect reconstruction of target sites from sequences of two half-sites resulting from two adjacent cuts of the nuclease of a library member nucleic acid (see e.g., Pattanayak, V. et al., *Nature Methods* 8, 765-770 (2011)). In contrast to such "two-cut" strategies, the methods of the present disclosure utilize a "one cut" screening strategy, which allows for the identification of library members that have been cut at least once by the nuclease. The "one-cut" selection strategies provided herein are compatible with single end high-throughput sequencing methods and do not require computational reconstruction of cleaved target sites from cut half-sites because they feature, in some embodiments, direct sequencing of an intact target nuclease sequence in a cut library member nucleic acid.

Additionally, the presently disclosed "one-cut" screening methods utilize concatemers of a candidate nuclease target site and constant insert region that are about 10-fold shorter than previously reported constructs used for two-cut strategies (~50 bp repeat sequence length versus ~500 bp repeat sequence length in previous reports). This difference in repeat sequence length in the concatemers of the library allows for the generation of highly complex libraries of candidate nuclease target sites, e.g., of libraries comprising 10^{12} different candidate nuclease target sequences. As described herein, an exemplary library of such complexity has been generated, templated on a known Cas9 nuclease target site by varying the sequence of the known target site. The exemplary library demonstrated that a greater than 10-fold coverage of all sequences with eight or fewer mutations of the known target site can be achieved using the strategies provided herein. The use of a shorter repeat sequence also allows the use of single-end sequencing, since both a cut half-site and an adjacent uncut site of the same library member are contained within a 100 nucleotide sequencing read.

The strategies, methods, libraries, and reagents provided herein can be utilized to analyze the sequence preferences and specificity of any site-specific nuclease, for example, to Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), homing endonucleases, organic compound nucleases, and enediyne antibiotics (e.g., dynemicin, neocarzinostatin, calicheamicin, esperamicin, bleomycin). Suitable nucleases in addition to the ones described herein will be apparent to those of skill in the art based on this disclosure.

Further, the methods, reagents, and strategies provided herein allow those of skill in the art to identify, design, and/or select nucleases with enhanced specificity and minimize the off-target effects of any given nuclease (e.g., site-specific nucleases such as ZFNs, and TALENS which produce cleavage products with sticky ends, as well as RNA-programmable nucleases, for example Cas9, which produce cleavage products having blunt ends). While of particular relevance to DNA and DNA-cleaving nucleases, the inventive concepts, methods, strategies, and reagents provided herein are not limited in this respect, but can be applied to any nucleic acid:nuclease pair.

Identifying Nuclease Target Sites Cleaved by a Site-Specific Nuclease

Some aspects of this disclosure provide improved methods and reagents to determine the nucleic acid target sites cleaved by any site-specific nuclease. The methods provided herein can be used for the evaluation of target site preferences and specificity of both nucleases that create blunt ends and nucleases that create sticky ends. In general, such methods comprise contacting a given nuclease with a library of target sites under conditions suitable for the nuclease to bind and cut a target site, and determining which target sites the nuclease actually cuts. A determination of a nuclease's target site profile based on actual cutting has the advantage over methods that rely on binding in that it measures a parameter more relevant for mediating undesired off-target effects of site-specific nucleases. In general, the methods provided herein comprise ligating an adapter of a known sequence to nucleic acid molecules that have been cut by a nuclease of interest via 5'-phosphate-dependent ligation. Accordingly, the methods provided herein are particularly useful for identifying target sites cut by nucleases that leave a phosphate moiety at the 5'-end of the cut nucleic acid strand when cleaving their target site. After ligating an adapter to the 5'-end of a cut nucleic acid strand, the cut strand can directly be sequenced using the adapter as a sequencing linker, or a part of the cut library member concatemer comprising an intact target site identical to the cut target site can be amplified via PCR and the amplification product can then be sequenced.

In some embodiments, the method comprises (a) providing a nuclease that cuts a double-stranded nucleic acid target site, wherein cutting of the target site results in cut nucleic acid strands comprising a 5'-phosphate moiety; (b) contacting the nuclease of (a) with a library of candidate nucleic acid molecules, wherein each nucleic acid molecule comprises a concatemer of a sequence comprising a candidate nuclease target site and a constant insert sequence, under conditions suitable for the nuclease to cut a candidate nucleic acid molecule comprising a target site of the nuclease; and (c) identifying nuclease target sites cut by the nuclease in (b) by determining the sequence of an uncut nuclease target site on the nucleic acid strand that was cut by the nuclease in step (b).

In some embodiments, the method comprises providing a nuclease and contacting the nuclease with a library of candidate nucleic acid molecules comprising candidate target sites. In some embodiments, the candidate nucleic acid molecules are double-stranded nucleic acid molecules. In some embodiments, the candidate nucleic acid molecules are DNA molecules. In some embodiments, each nucleic acid molecule in the library comprises a concatemer of a sequence comprising a candidate nuclease target site and a constant insert sequence. For example, in some embodiments, the library comprises nucleic acid molecules that comprise the structure

R_1-[(candidate nuclease target site)-(constant insert sequence)]$_n$-R_2, wherein R_1 and R_2 are, independently, nucleic acid sequences that may comprise a fragment of the [(candidate nuclease target site)-(constant insert sequence)] structure, and n is an integer between 2 and y. In some embodiments, y is at least 10^1, at least 10^2, at least 10^3, at least 10^4, at least 10^5, at least 10^6, at least 10^7, at least 10^8, at least 10^9, at least 10^{10}, at least 10^{11}, at least 10^{12}, at least 10^{13}, at least 10^{14}, or at least 10^{15}. In some embodiments, y is less than 10^2, less than 10^3, less than 10^4, less than 10^5, less than 10^6, less than 10^7, less than 10^8, less than 10^9, less than 10^{10}, less than 10^{11}, less than 10^{12}, less than 10^{13}, less than 10^{14}, or less than 10^{15}

For example, in some embodiments, the candidate nucleic acid molecules of the library comprise a candidate nuclease target site of the structure [(N_Z)-(PAM)], and, thus, the nucleic acid molecules of the library comprise the structure R_1—[(N_Z)-(PAM)-(constant region)]$_X$-R_2, wherein R_1 and R_2 are, independently, nucleic acid sequences that may comprise a fragment of the [(N_Z)-(PAM)-(constant region)] repeat unit; each N represents, independently, any nucleotide; Z is an integer between 1 and 50; and X is an integer between 2 and y. In some embodiments, y is at least 10^1, at least 10^2, at least 10^3, at least 10^4, at least 10^5, at least 10^6, at least 10^7, at least 10^8, at least 10^9, at least 10^{10}, at least 10^{11}, at least 10^{12}, at least 10^{13}, at least 10^{14}, or at least 10^{15}. In some embodiments, y is less than 10^2, less than 10^3, less than 10^4, less than 10^5, less than 10^6, less than 10^7, less than 10^8, less than 10^9, less than 10^{10}, less than 10^{11}, less than 10^{12}, less than 10^{13}, less than 10^{14}, or less than 10^{15}. In some embodiments, Z is at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 25, at least 30, at least 35, at least 40, at least 45, or at least 50. In some embodiments, Z is 20. Each N represents, independently, any nucleotide. Accordingly, a sequence provided as N_Z with $_Z$=2 would be NN, with each N, independently, representing A, T, G, or C. Accordingly, N_Z with $_Z$=2 can represent AA, AT, AG, AC, TA, TT, TG, TC, GA, GT, GG, GC, CA, CT, CG, and CC.

In other embodiments, the candidate nucleic acid molecules of the library comprise a candidate nuclease target site of the structure [left-half site]-[spacer sequence]-[right-half site] ("LSR"), and, thus, the nucleic acid molecules of the library comprise the structure R_1-[(LSR)-(constant region)]$_X$-R_2, wherein R_1 and R_2 are, independently, nucleic acid sequences that may comprise a fragment of the [(LSR)-(constant region)] repeat unit, and X is an integer between 2 and y. In some embodiments, y is at least 10^1, at least 10^2, at least 10^3, at least 10^4, at least 10^5, at least 10^6, at least 10^7, at least 10^8, at least 10^9, at least 10^{10}, at least 10^{11}, at least 10^{12}, at least 10^{13}, at least 10^{14}, or at least 10^{15}. In some embodiments, y is less than 10^2, less than 10^3, less than 10^4, less than 10^5, less than 10^6, less than 10^7, less than 10^8, less than 10^9, less than 10^{10}, less than 10^{11}, less than 10^{12}, less than 10^{13}, less than 10^{14}, or less than 10^{15}. The constant region, in some embodiments, is of a length that allows for efficient self-ligation of a single repeat unit. Suitable lengths will be apparent to those of skill in the art. For example, in some embodiments, the constant region is between 5 and 100 base pairs long, for example, about 5 base pairs, about 10 base pairs, about 15 base pairs, about 20 base pairs, about 25 base pairs, about 30 base pairs, about 35 base pairs, about 40 base pairs, about 50 base pairs, about 60 base pairs, about 70 base pairs, about 80 base pairs, about 90 base pairs, or about 100 base pairs long. In some embodiments, the constant region is 16 base pairs long. In some embodiments, the nuclease cuts a double-stranded nucleic acid target site and creates blunt ends. In other embodiments, the nuclease creates a 5'-overhang. In some such embodiments, the target site comprises a [left-half site]-[spacer sequence]-[right-half site] (LSR) structure, and the nuclease cuts the target site within the spacer sequence.

In some embodiments, a nuclease cuts a double-stranded target site and creates blunt ends. In some embodiments, a nuclease cuts a double-stranded target site and creates an overhang, or sticky end, for example, a 5'-overhang. In some such embodiments, the method comprises filling in the 5'-overhangs of nucleic acid molecules produced from a nucleic acid molecule that has been cut once by the nuclease, wherein the nucleic acid molecules comprise a constant insert sequence flanked by a left or right half-site and cut spacer sequence on one side, and an uncut target site sequence on the other side, thereby creating blunt ends.

In some embodiments, the determining of step (c) comprises ligating a first nucleic acid adapter to the 5' end of a nucleic acid strand that was cut by the nuclease in step (b) via 5'-phosphate-dependent ligation. In some embodiments, the nuclease creates blunt ends. In such embodiments, an adapter can directly be ligated to the blunt ends resulting from the nuclease cut of the target site by contacting the cut library members with a double-stranded, blunt-ended adapter lacking 5' phosphorylation. In some embodiments, the nuclease creates an overhang (sticky end). In some such embodiments, an adapter may be ligated to the cut site by contacting the cut library member with an excess of adapter having a compatible sticky end. If a nuclease is used that cuts within a constant spacer sequence between variable half-sites, the sticky end can be designed to match the 5' overhang created from the spacer sequence. In embodiments, where the nuclease cuts within a variable sequence, a population of adapters having a variable overhang sequence and a constant annealed sequence (for use as a sequencing linker or PCR primer) may be used, or the 5' overhangs may be filled in to form blunt ends before adapter ligation.

In some embodiments, the determining of step (c) further comprises amplifying a fragment of the concatemer cut by the nuclease that comprises an uncut target site via PCR using a PCR primer that hybridizes with the adapter and a PCR primer that hybridizes with the constant insert sequence. Typically, the amplification of concatemers via PCR will yield amplicons comprising at least one intact candidate target site identical to the cut target sites because the target sites in each concatemer are identical. For single-direction sequencing, an enrichment of amplicons that comprise one intact target site, no more than two intact target sites, no more than three intact target sites, no more than four intact target sites, or no more than five intact target sites may be desirable. In embodiments where PCR is used for amplification of cut nucleic acid molecules, the PCR parameters can be optimized to favor the amplification of short sequences and disfavor the amplification of longer sequences, e.g., by using a short elongation time in the PCR cycle. Another possibility for enrichment of short amplicons is size fractionation, e.g., via gel electrophoresis or size exclusion chromatography. Size fractionation can be performed before and/or after amplification. Other suitable methods for enrichment of short amplicons will be apparent to those of skill in the art and the disclosure is not limited in this respect.

In some embodiments, the determining of step (c) comprises sequencing the nucleic acid strand that was cut by the nuclease in step (b), or a copy thereof obtained via amplifi-

cation, e.g., by PCR. Sequencing methods are well known to those of skill in the art. The disclosure is not limited in this respect.

In some embodiments, the nuclease being profiled using the inventive system is an RNA-programmable nuclease that forms a complex with an RNA molecule, and wherein the nuclease:RNA complex specifically binds a nucleic acid sequence complementary to the sequence of the RNA molecule. In some embodiments, the RNA molecule is a single-guide RNA (sgRNA). In some embodiments, the sgRNA comprises 5-50 nucleotides, 10-30 nucleotides, 15-25 nucleotides, 18-22 nucleotides, 19-21 nucleotides, e.g., 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides. In some embodiments, the sgRNA comprises 5-50 nucleotides, 10-30 nucleotides, 15-25 nucleotides, 18-22 nucleotides, 19-21 nucleotides, e.g., 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides that are complementary to a sequence of the nuclease target site. In some embodiments, the sgRNA comprises 20 nucleotides that are complementary to the nuclease target site. In some embodiments, the nuclease is a Cas9 nuclease. In some embodiments, the nuclease target site comprises a [sgRNA-complementary sequence]-[protospacer adjacent motif (PAM)] structure, and the nuclease cuts the target site within the sgRNA-complementary sequence. In some embodiments, the sgRNA-complementary sequence comprises 5-50 nucleotides, 10-30 nucleotides, 15-25 nucleotides, 18-22 nucleotides, 19-21 nucleotides, e.g., 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides.

In some embodiments, the RNA-programmable nuclease is a Cas9 nuclease. The RNA-programmable Cas9 endonuclease cleaves double-stranded DNA (dsDNA) at sites adjacent to a two-base-pair PAM motif and complementary to a guide RNA sequence (sgRNA). Typically, the sgRNA sequence that is complementary to the target site sequence is about 20 nucleotides long, but shorter and longer complementary sgRNA sequences can be used as well. For example, in some embodiments, the sgRNA comprises 5-50 nucleotides, 10-30 nucleotides, 15-25 nucleotides, 18-22 nucleotides, 19-21 nucleotides, e.g., 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides. The Cas9 system has been used to modify genomes in multiple cell types, demonstrating its potential as a facile genome-engineering tool.

In some embodiments, the nuclease comprises an unspecific nucleic acid cleavage domain. In some embodiments, the nuclease comprises a FokI cleavage domain. In some embodiments, the nuclease comprises a nucleic acid cleavage domain that cleaves a target sequence upon cleavage domain dimerization. In some embodiments, the nuclease comprises a binding domain that specifically binds a nucleic acid sequence. In some embodiments, the binding domain comprises a zinc finger. In some embodiments, the binding domain comprises at least 2, at least 3, at least 4, or at least 5 zinc fingers. In some embodiments, the nuclease is a Zinc Finger Nuclease. In some embodiments, the binding domain comprises a Transcriptional Activator-Like Element. In some embodiments, the nuclease is a Transcriptional Activator-Like Element Nuclease (TALEN). In some embodiments, the nuclease is a homing endonuclease. In some embodiments, the nuclease is an organic compound. In some embodiments, the nuclease comprises an enediyne functional group. In some embodiments, the nuclease is an antibiotic. In some embodiments, the compound is dynemicin, neocarzinostatin, calicheamicin, esperamicin, bleomycin, or a derivative thereof.

Incubation of the nuclease with the library nucleic acids will result in cleavage of those concatemers in the library that comprise target sites that can be bound and cleaved by the nuclease. If a given nuclease cleaves a specific target site with high efficiency, a concatemer comprising target sites will be cut, e.g., once or multiple times, resulting in the generation of fragments comprising a cut target site adjacent to one or more repeat units. Depending on the structure of the library members, an exemplary cut nucleic acid molecule released from a library member concatemer by a single nuclease cleavage may, for example, be of the structure (cut target site)-(constant region)-$[(\text{target site})\text{-}(\text{constant region})]_X\text{-}R_2$. For example, in the context of an RNA-guided nuclease, an exemplary cut nucleic acid molecule released from a library member concatemer by a single nuclease cleavage may, for example, be of the structure (PAM)-(constant region)-$[(N_Z)\text{-}(\text{PAM})\text{-}(\text{constant region})]_X\text{-}R_2$. And in the context of a nuclease cutting an LSR structure within the spacer region, an exemplary cut nucleic acid molecule released from a library member concatemer by a single nuclease cleavage may, for example, be of the structure (cut spacer region)-(right half site)-(constant region)-$[(\text{LSR})\text{-}(\text{constant region})]_X\text{-}R_2$. Such cut fragments released from library candidate molecules can then be isolated and/or the sequence of the target site cleaved by the nuclease identified by sequencing an intact target site (e.g., an intact (N_Z)-(PAM) site of released repeat units. See, e.g., FIG. **1**B for an illustration.

Suitable conditions for exposure of the library of nucleic acid molecules will be apparent to those of skill in the art. In some embodiments, suitable conditions do not result in denaturation of the library nucleic acids or the nuclease and allow for the nuclease to exhibit at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 98% of its nuclease activity.

Additionally, if a given nuclease cleaves a specific target site, some cleavage products will comprise a cut half site and an intact, or uncut target site. As described herein, such products can be isolated by routine methods, and because the insert sequence, in some aspects, is less than 100 base pairs, such isolated cleavage products may be sequenced in a single read-through, allowing identification of the target site sequence without reconstructing the sequence, e.g., from cut half sites.

Any method suitable for isolation and sequencing of the repeat units can be employed to elucidate the LSR sequence cleaved by the nuclease. For example, since the length of the constant region is known, individual released repeat units can be separated based on their size from the larger uncut library nucleic acid molecules as well as from fragments of library nucleic acid molecules that comprise multiple repeat units (indicating non-efficient targeted cleavage by the nuclease). Suitable methods for separating and/or isolating nucleic acid molecules based on their size are well-known to those of skill in the art and include, for example, size fractionation methods, such as gel electrophoresis, density gradient centrifugation, and dialysis over a semi-permeable membrane with a suitable molecular cutoff value. The separated/isolated nucleic acid molecules can then be further characterized, for example, by ligating PCR and/or sequencing adapters to the cut ends and amplifying and/or sequencing the respective nucleic acids. Further, if the length of the constant region is selected to favor self-ligation of individual released repeat units, such individual released repeat units may be enriched by contacting the nuclease treated library molecules with a ligase and subsequent amplification and/or sequencing based on the circularized nature of the self-ligated individual repeat units.

In some embodiments, where a nuclease is used that generates 5'-overhangs as a result of cutting a target nucleic acid, the 5'-overhangs of the cut nucleic acid molecules are filled in. Methods for filling in 5'-overhangs are well known to those of skill in the art and include, for example, methods using DNA polymerase I Klenow fragment lacking exonuclease activity (Klenow (3'->5' exo-)). Filling in 5'-overhangs results in the overhang-templated extension of the recessed strand, which, in turn, results in blunt ends. In the case of single repeat units released from library concatemers, the resulting structure is a blunt-ended S_2'R-(constant region)-LS_1', with S_1' and S_2' comprising blunt ends. PCR and/or sequencing adapters can then be added to the ends by blunt end ligation and the respective repeat units (including S_2'R and LS_1' regions) can be sequenced. From the sequence data, the original LSR region can be deduced. Blunting of the overhangs created during the nuclease cleavage process also allows for distinguishing between target sites that were properly cut by the respective nuclease and target sites that were non-specifically cut, e.g., based on non-nuclease effects such as physical shearing. Correctly cleaved nuclease target sites can be recognized by the existence of complementary S_2'R and LS_1' regions, which comprise a duplication of the overhang nucleotides as a result of the overhang fill in while target sites that were not cleaved by the respective nuclease are unlikely to comprise overhang nucleotide duplications. In some embodiments, the method comprises identifying the nuclease target site cut by the nuclease by determining the sequence of the left-half site, the right-half-site, and/or the spacer sequence of a released individual repeat unit. Any suitable method for amplifying and/or sequencing can be used to identify the LSR sequence of the target site cleaved by the respective nuclease. Methods for amplifying and/or sequencing nucleic acids are well known to those of skill in the art and the disclosure is not limited in this respect. In the case of nucleic acids released from library concatemers that comprise a cut half site and an uncut target site (e.g., comprises at least about 1.5 repeat sequences), filling in the 5'-overhangs also provides for assurance that the nucleic acid was cleaved by the nuclease. Because the nucleic acid also comprises an intact, or uncut target site, the sequence of said site can be determined without having to reconstruct the sequence from a left-half site, right-half site, and/or spacer sequence.

Some of the methods and strategies provided herein allow for the simultaneous assessment of a plurality of candidate target sites as possible cleavage targets for any given nuclease. Accordingly, the data obtained from such methods can be used to compile a list of target sites cleaved by a given nuclease, which is also referred to herein as a target site profile. If a sequencing method is used that allows for the generation of quantitative sequencing data, it is also possible to record the relative abundance of any nuclease target site detected to be cleaved by the respective nuclease. Target sites that are cleaved more efficiently by the nuclease will be detected more frequently in the sequencing step, while target sites that are not cleaved efficiently will only rarely release an individual repeat unit from a candidate concatemer, and thus, will only generate few, if any, sequencing reads. Such quantitative sequencing data can be integrated into a target site profile to generate a ranked list of highly preferred and less preferred nuclease target sites.

The methods and strategies of nuclease target site profiling provided herein can be applied to any site-specific nuclease, including, for example, ZFNs, TALENs, homing endonucleases, and RNA-programmable nucleases, such as Cas9 nucleases. As described in more detail herein, nuclease specificity typically decreases with increasing nuclease concentration, and the methods described herein can be used to determine a concentration at which a given nuclease efficiently cuts its intended target site, but does not efficiently cut any off-target sequences. In some embodiments, a maximum concentration of a therapeutic nuclease is determined at which the therapeutic nuclease cuts its intended nuclease target site but does not cut more than 10, more than 5, more than 4, more than 3, more than 2, more than 1, or any additional sites. In some embodiments, a therapeutic nuclease is administered to a subject in an amount effective to generate a final concentration equal or lower than the maximum concentration determined as described above.

In some embodiments, the library of candidate nucleic acid molecules used in the methods provided herein comprises at least 10^8, at least 10^9, at least 10^{10}, at least 10^{11}, or at least 10^{12} different candidate nuclease target sites.

In some embodiments, the nuclease is a therapeutic nuclease which cuts a specific nuclease target site in a gene associated with a disease. In some embodiments, the method further comprises determining a maximum concentration of the therapeutic nuclease at which the therapeutic nuclease cuts the specific nuclease target site and does not cut more than 10, more than 5, more than 4, more than 3, more than 2, more than 1, or no additional sites. In some embodiments, the method further comprises administering the therapeutic nuclease to a subject in an amount effective to generate a final concentration equal or lower than the maximum concentration.

Nuclease Target Site Libraries

Some embodiments of this disclosure provide libraries of nucleic acid molecules for nuclease target site profiling. In some embodiments, the candidate nucleic acid molecules of the library comprise the structure R_1—[(N_Z)-(PAM)-(constant region)]$_X$-R_2, wherein R_1 and R_2 are, independently, nucleic acid sequences that may comprise a fragment of the [(N_Z)-(PAM)-(constant region)] repeat unit; each N represents, independently, any nucleotide; Z is an integer between 1 and 50; and X is an integer between 2 and y. In some embodiments, y is at least 10^1, at least 10^2, at least 10^3, at least 10^4, at least 10^5, at least 10^6, at least 10^7, at least 10^8, at least 10^9, at least 10^{10}, at least 10^{11}, at least 10^{12}, at least 10^{13}, at least 10^{14}, or at least 10^{15}. In some embodiments, y is less than 10^2, less than 10^3, less than 10^4, less than 10^5, less than 10^6, less than 10^7, less than 10^8, less than 10^9, less than 10^{10}, less than 10^{11}, less than 10^{12}, less than 10^{13}, less than 10^{14}, or less than 10^{15}. In some embodiments, Z is at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 25, at least 30, at least 35, at least 40, at least 45, or at least 50. In some embodiments, Z is 20. Each N represents, independently, any nucleotide. Accordingly, a sequence provided as N_Z with $_Z$=2 would be NN, with each N, independently, representing A, T, G, or C. Accordingly, N_Z with $_Z$=2 can represent AA, AT, AG, AC, TA, TT, TG, TC, GA, GT, GG, GC, CA, CT, CG, and CC.

In some embodiments, a library is provided comprising candidate nucleic acid molecules that comprise target sites with a partially randomized left-half site, a partially randomized right-half site, and/or a partially randomized spacer sequence. In some embodiments, the library is provided comprising candidate nucleic acid molecules that comprise target sites with a partially randomized left half site, a fully randomized spacer sequence, and a partially randomized right half site. In some embodiments, a library is provided comprising candidate nucleic acid molecules that comprise target sites with a partially or fully randomized sequence, wherein the

target sites comprise the structure [N_Z-(PAM)], for example as described herein. In some embodiments, partially randomized sites differ from the consensus site by more than 5%, more than 10%, more than 15%, more than 20%, more than 25%, or more than 30% on average, distributed binomially.

In some embodiments such a library comprises a plurality of nucleic acid molecules, each comprising a concatemer of a candidate nuclease target site and a constant insert sequence, also referred to herein as a constant region. For example, in some embodiments, the candidate nucleic acid molecules of the library comprise the structure R_1-[(sgRNA-complementary sequence)-(PAM)-(constant region)]$_X$-R_2, or the structure R_1-[(LSR)-(constant region)]$_X$-R_2, wherein the structure in square brackets ("[. . .]") is referred to as a repeat unit or repeat sequence; R_1 and R_2 are, independently, nucleic acid sequences that may comprise a fragment of the repeat unit, and X is an integer between 2 and y. In some embodiments, y is at least 10^1, at least 10^2, at least 10^3, at least 10^4, at least 10^5, at least 10^6, at least 10^7, at least 10^8, at least 10^9, at least 10^{10}, at least 10^{11}, at least 10^{12}, at least 10^{13}, at least 10^{14}, or at least 10^{15}. In some embodiments, y is less than 10^2, less than 10^3, less than 10^4, less than 10^5, less than 10^6, less than 10^7, less than 10^8, less than 10^9, less than 10^{10}, less than 10^{11}, less than 10^{12}, less than 10^{13}, less than 10^{14}, or less than 10^{15}. The constant region, in some embodiments, is of a length that allows for efficient self-ligation of a single repeat unit. In some embodiments, the constant region is of a length that allows for efficient separation of single repeat units from fragments comprising two or more repeat units. In some embodiments, the constant region is of a length allows for efficient sequencing of a complete repeat unit in one sequencing read. Suitable lengths will be apparent to those of skill in the art. For example, in some embodiments, the constant region is between 5 and 100 base pairs long, for example, about 5 base pairs, about 10 base pairs, about 15 base pairs, about 20 base pairs, about 25 base pairs, about 30 base pairs, about 35 base pairs, about 40 base pairs, about 50 base pairs, about 60 base pairs, about 70 base pairs, about 80 base pairs, about 90 base pairs, or about 100 base pairs long. In some embodiments, the constant region is 1, 2, 3, 4, 5, 6, 7, 8, 9, 0, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, or 80 base pairs long.

An LSR site typically comprises a [left-half site]-[spacer sequence]-[right-half site] structure. The lengths of the half-size and the spacer sequence will depend on the specific nuclease to be evaluated. In general, the half-sites will be 6-30 nucleotides long, and preferably 10-18 nucleotides long. For example, each half site individually may be 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides long. In some embodiments, an LSR site may be longer than 30 nucleotides. In some embodiments, the left half site and the right half site of an LSR are of the same length. In some embodiments, the left half site and the right half site of an LSR are of different lengths. In some embodiments, the left half site and the right half site of an LSR are of different sequences. In some embodiments, a library is provided that comprises candidate nucleic acids which comprise LSRs that can be cleaved by a FokI cleavage domain, a Zinc Finger Nuclease (ZFN), a Transcription Activator-Like Effector Nuclease (TALEN), a homing endonuclease, or an organic compound (e.g., an enediyne antibiotic such as dynemicin, neocarzinostatin, calicheamicin, and esperamicinl; and bleomycin).

In some embodiments, a library of candidate nucleic acid molecules is provided that comprises at least 10^5, at least 10^6, at least 10^7, at least 10^8, at least 10^9, at least 10^{10}, at least 10^{11}, at least 10^{12}, at least 10^{13}, at least 10^{14}, or at least 10^{15} different candidate nuclease target sites. In some embodiments, the candidate nucleic acid molecules of the library are concatemers produced from a secularized templates by rolling cycle amplification. In some embodiments, the library comprises nucleic acid molecules, e.g., concatemers, of a molecular weight of at least 5 kDa, at least 6 kDa, at least 7 kDa, at least 8 kDa, at least 9 kDa, at least 10 kDa, at least 12 kDa, or at least 15 kDa. In some embodiments, the molecular weight of the nucleic acid molecules within the library may be larger than 15 kDa. In some embodiments, the library comprises nucleic acid molecules within a specific size range, for example, within a range of 5-7 kDa, 5-10 kDa, 8-12 kDa, 10-15 kDa, or 12-15 kDa, or 5-10 kDa or any possible sub-range. While some methods suitable for generating nucleic acid concatemers according to some aspects of this disclosure result in the generation of nucleic acid molecules of greatly different molecular weights, such mixtures of nucleic acid molecules may be size fractionated to obtain a desired size distribution. Suitable methods for enriching nucleic acid molecules of a desired size or excluding nucleic acid molecules of a desired size are well known to those of skill in the art and the disclosure is not limited in this respect.

In some embodiments, partially randomized sites differ from the consensus site by no more than 10%, no more than 15%, no more than 20%, no more than 25%, nor more than 30%, no more than 40%, or no more than 50% on average, distributed binomially. For example, in some embodiments partially randomized sites differ from the consensus site by more than 5%, but by no more than 10%; by more than 10%, but by no more than 20%; by more than 20%, but by no more than 25%; by more than 5%, but by no more than 20%, and so on. Using partially randomized nuclease target sites in the library is useful to increase the concentration of library members comprising target sites that are closely related to the consensus site, for example, that differ from the consensus sites in only one, only two, only three, only four, or only five residues. The rationale behind this is that a given nuclease, for example a given ZFN or RNA-programmable nuclease, is likely to cut its intended target site and any closely related target sites, but unlikely to cut a target sites that is vastly different from or completely unrelated to the intended target site. Accordingly, using a library comprising partially randomized target sites can be more efficient than using libraries comprising fully randomized target sites without compromising the sensitivity in detecting any off-target cleavage events for any given nuclease. Thus, the use of partially randomized libraries significantly reduces the cost and effort required to produce a library having a high likelihood of covering virtually all off-target sites of a given nuclease. In some embodiments however it may be desirable to use a fully randomized library of target sites, for example, in embodiments, where the specificity of a given nuclease is to be evaluated in the context of any possible site in a given genome.

Selection and Design of Site-Specific Nucleases

Some aspects of this disclosure provide methods and strategies for selecting and designing site-specific nucleases that allow the targeted cleavage of a single, unique sites in the context of a complex genome. In some embodiments, a method is provided that comprises providing a plurality of candidate nucleases that are designed or known to cut the same consensus sequence; profiling the target sites actually cleaved by each candidate nuclease, thus detecting any cleaved off-target sites (target sites that differ from the con-

sensus target site); and selecting a candidate nuclease based on the off-target site(s) so identified. In some embodiments, this method is used to select the most specific nuclease from a group of candidate nucleases, for example, the nuclease that cleaves the consensus target site with the highest specificity, the nuclease that cleaves the lowest number of off-target sites, the nuclease that cleaves the lowest number of off-target sites in the context of a target genome, or a nuclease that does not cleave any target site other than the consensus target site. In some embodiments, this method is used to select a nuclease that does not cleave any off-target site in the context of the genome of a subject at concentration that is equal to or higher than a therapeutically effective concentration of the nuclease.

The methods and reagents provided herein can be used, for example, to evaluate a plurality of different nucleases targeting the same intended targets site, for example, a plurality of variations of a given site-specific nuclease, for example a given zinc finger nuclease. Accordingly, such methods may be used as the selection step in evolving or designing a novel site-specific nucleases with improved specificity.

Identifying Unique Nuclease Target Sites within a Genome

Some embodiments of this disclosure provide a method for selecting a nuclease target site within a genome. As described in more detail elsewhere herein, it was surprisingly discovered that off target sites cleaved by a given nuclease are typically highly similar to the consensus target site, e.g., differing from the consensus target site in only one, only two, only three, only four, or only five nucleotide residues. Based on this discovery, a nuclease target sites within the genome can be selected to increase the likelihood of a nuclease targeting this site not cleaving any off target sites within the genome. For example, in some embodiments, a method is provided that comprises identifying a candidate nuclease target site; and comparing the candidate nuclease target site to other sequences within the genome. Methods for comparing candidate nuclease target sites to other sequences within the genome are well known to those of skill in the art and include for example sequence alignment methods, for example, using a sequence alignment software or algorithm such as BLAST on a general purpose computer. A suitable unique nuclease target site can then be selected based on the results of the sequence comparison. In some embodiments, if the candidate nuclease target site differs from any other sequence within the genome by at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or at least 10 nucleotides, the nuclease target site is selected as a unique site within the genome, whereas if the site does not fulfill this criteria, the site may be discarded. In some embodiments, once a site is selected based on the sequence comparison, as outlined above, a site-specific nuclease targeting the selected site is designed. For example, a zinc finger nuclease may be designed to target any selected nuclease target site by constructing a zinc finger array binding the target site, and conjugating the zinc finger array to a DNA cleavage domain. In embodiments where the DNA cleavage domain needs to dimerize in order to cleave DNA, to zinc finger arrays will be designed, each binding a half site of the nuclease target site, and each conjugated to a cleavage domain. In some embodiments, nuclease designing and/or generating is done by recombinant technology. Suitable recombinant technologies are well known to those of skill in the art, and the disclosure is not limited in this respect.

In some embodiments, a site-specific nuclease designed or generated according to aspects of this disclosure is isolated and/or purified. The methods and strategies for designing site-specific nucleases according to aspects of this disclosure can be applied to design or generate any site-specific nuclease, including, but not limited to Zinc Finger Nucleases, Transcription Activator-Like Effector Nucleases (TALENs), a homing endonuclease, an organic compound nuclease, or an enediyne antibiotic (e.g., dynemicin, neocarzinostatin, calicheamicin, esperamicin, bleomycin).

Isolated Nucleases

Some aspects of this disclosure provide isolated site-specific nucleases with enhanced specificity that are designed using the methods and strategies described herein. Some embodiments, of this disclosure provide nucleic acids encoding such nucleases. Some embodiments of this disclosure provide expression constructs comprising such encoding nucleic acids. For example, in some embodiments an isolated nuclease is provided that has been engineered to cleave a desired target site within a genome, and has been evaluated according to a method provided herein to cut less than 1, less than 2, less than 3, less than 4, less than 5, less than 6, less than 7, less than 8, less than 9 or less than 10 off-target sites at a concentration effective for the nuclease to cut its intended target site. In some embodiments an isolated nuclease is provided that has been engineered to cleave a desired unique target site that has been selected to differ from any other site within a genome by at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or at least 10 nucleotide residues. In some embodiments, the isolated nuclease is an RNA-programmable nuclease, such as a Cas9 nuclease; a Zinc Finger Nuclease (ZFN); or a Transcription Activator-Like Effector Nuclease (TALEN), a homing endonuclease, an organic compound nuclease, or an enediyne antibiotic (e.g., dynemicin, neocarzinostatin, calicheamicin, esperamicin, bleomycin). In some embodiments, the isolated nuclease cleaves a target site within an allele that is associated with a disease or disorder. In some embodiments, the isolated nuclease cleaves a target site the cleavage of which results in treatment or prevention of a disease or disorder. In some embodiments, the disease is HIV/AIDS, or a proliferative disease. In some embodiments, the allele is a CCR5 (for treating HIV/AIDS) or a VEGFA allele (for treating a proliferative disease).

In some embodiments, the isolated nuclease is provided as part of a pharmaceutical composition. For example, some embodiments provide pharmaceutical compositions comprising a nuclease as provided herein, or a nucleic acid encoding such a nuclease, and a pharmaceutically acceptable excipient. Pharmaceutical compositions may optionally comprise one or more additional therapeutically active substances.

In some embodiments, compositions provided herein are administered to a subject, for example, to a human subject, in order to effect a targeted genomic modification within the subject. In some embodiments, cells are obtained from the subject and contacted with a nuclease or a nuclease-encoding nucleic acid ex vivo, and re-administered to the subject after the desired genomic modification has been effected or detected in the cells. Although the descriptions of pharmaceutical compositions provided herein are principally directed to pharmaceutical compositions which are suitable for administration to humans, it will be understood by the skilled artisan that such compositions are generally suitable for administration to animals of all sorts. Modification of pharmaceutical compositions suitable for administration to humans in order to render the compositions suitable for administration to various animals is well understood, and the ordinarily skilled veterinary pharmacologist can design and/or perform such modification with merely ordinary, if any, experimentation. Subjects to which administration of the pharmaceutical compositions is contemplated include, but are not limited to, humans and/or other primates; mammals,

including commercially relevant mammals such as cattle, pigs, horses, sheep, cats, dogs, mice, and/or rats; and/or birds, including commercially relevant birds such as chickens, ducks, geese, and/or turkeys.

Formulations of the pharmaceutical compositions described herein may be prepared by any method known or hereafter developed in the art of pharmacology. In general, such preparatory methods include the step of bringing the active ingredient into association with an excipient and/or one or more other accessory ingredients, and then, if necessary and/or desirable, shaping and/or packaging the product into a desired single- or multi-dose unit.

Pharmaceutical formulations may additionally comprise a pharmaceutically acceptable excipient, which, as used herein, includes any and all solvents, dispersion media, diluents, or other liquid vehicles, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, solid binders, lubricants and the like, as suited to the particular dosage form desired. Remington's *The Science and Practice of Pharmacy,* 21st Edition, A. R. Gennaro (Lippincott, Williams & Wilkins, Baltimore, Md., 2006; incorporated in its entirety herein by reference) discloses various excipients used in formulating pharmaceutical compositions and known techniques for the preparation thereof. See also PCT application PCT/US2010/055131, incorporated in its entirety herein by reference, for additional suitable methods, reagents, excipients and solvents for producing pharmaceutical compositions comprising a nuclease. Except insofar as any conventional excipient medium is incompatible with a substance or its derivatives, such as by producing any undesirable biological effect or otherwise interacting in a deleterious manner with any other component (s) of the pharmaceutical composition, its use is contemplated to be within the scope of this disclosure.

The function and advantage of these and other embodiments of the present invention will be more fully understood from the Examples below. The following Examples are intended to illustrate the benefits of the present invention and to describe particular embodiments, but are not intended to exemplify the full scope of the invention. Accordingly, it will be understood that the Examples are not meant to limit the scope of the invention.

EXAMPLES

Materials and Methods

Oligonucleotides. All oligonucleotides used in this study were purchased from Integrated DNA Technologies. Oligonucleotide sequences are listed in Table 9.

Expression and Purification of *S. pyogenes* Cas9. *E. coli* Rosetta (DE3) cells were transformed with plasmid pMJ806[11], encoding the *S. pyogenes* cas9 gene fused to an N-terminal 6× His-tag/maltose binding protein. The resulting expression strain was inoculated in Luria-Bertani (LB) broth containing 100 μg/mL of ampicillin and 30 μg/mL of chloramphenicol at 37° C. overnight. The cells were diluted 1:100 into the same growth medium and grown at 37° C. to OD_{600}~0.6. The culture was incubated at 18° C. for 30 min, and isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at 0.2 mM to induce Cas9 expression. After ~17 h, the cells were collected by centrifugation at 8,000 g and resuspended in lysis buffer (20 mM tris(hydroxymethyl)-aminomethane (Tris)-HCl, pH 8.0, 1 M KCl, 20% glycerol, 1 mM tris(2-carboxyethyl)phosphine (TCEP)). The cells were lysed by sonication (10 sec pulse-on and 30 sec pulse-off for 10 min total at 6 W output) and the soluble lysate was obtained by centrifugation at 20,000 g for 30 min. The cell lysate was incubated with nickel-nitriloacetic acid (nickel-NTA) resin (Qiagen) at 4° C. for 20 min to capture His-tagged Cas9. The resin was transferred to a 20-mL column and washed with 20 column volumes of lysis buffer. Cas9 was eluted in 20 mM Tris-HCl (pH 8), 0.1 M KCl, 20% glycerol, 1 mM TCEP, and 250 mM imidazole, and concentrated by Amicon ultra centrifugal filter (Millipore, 30-kDa molecular weight cut-off) to ~50 mg/mL. The 6× His tag and maltose-binding protein were removed by TEV protease treatment at 4° C. for 20 h and captured by a second Ni-affinity purification step. The eluent, containing Cas9, was injected into a HiTrap SP FF column (GE Healthcare) in purification buffer containing 20 mM Tris-HCl (pH 8), 0.1 M KCl, 20% glycerol, and 1 mM TCEP. Cas9 was eluted with purification buffer containing a linear KCl gradient from 0.1 M to 1 M over five column volumes. The eluted Cas9 was further purified by a HiLoad Superdex 200 column in purification buffer, snap-frozen in liquid nitrogen, and stored in aliquots at −80° C.

In Vitro RNA Transcription. 100 pmol CLTA(#) v2.1 fwd and v2.1 template rev were incubated at 95° C. and cooled at 0.1° C./s to 37° C. in NEBuffer2 (50 mM sodium chloride, 10 mM Tris-HCl, 10 mM magnesium chloride, 1 mM dithiothreitol, pH 7.9) supplemented with 10 μM dNTP mix (Bio-Rad). 10 U of Klenow Fragment (3'→5' exo⁻) (NEB) were added to the reaction mixture and a double-stranded CLTA(#) v2.1 template was obtained by overlap extension for 1 h at 37° C. 200 nM CLTA(#) v2.1 template alone or 100 nM CLTA(#) template with 100 nM T7 promoter oligo was incubated overnight at 37° C. with 0.16 U/μL of T7 RNA Polymerase (NEB) in NEB RNAPol Buffer (40 mM Tris-HCl, pH 7.9, 6 mM magnesium chloride, 10 mM dithiothreitol, 2 mM spermidine) supplemented with 1 mM rNTP mix (1 mM rATP, 1 mM rCTP, 1 mM rGTP, 1 mM rUTP). In vitro transcribed RNA was precipitated with ethanol and purified by gel electrophoresis on a Criterion 10% polyacrylamide TBE-Urea gel (Bio-Rad). Gel-purified sgRNA was precipitated with ethanol and redissolved in water.

In Vitro Library Construction. 10 pmol of CLTA(#) lib oligonucleotides were separately circularized by incubation with 100 units of CircLigase II ssDNA Ligase (Epicentre) in 1× CircLigase II Reaction Buffer (33 mM Tris-acetate, 66 mM potassium acetate, 0.5 mM dithiothreitol, pH 7.5) supplemented with 2.5 mM manganese chloride in a total reaction volume of 20 μL for 16 hours at 60° C. The reaction mixture was incubated for 10 minutes at 85° C. to inactivate the enzyme. 5 μL (5 pmol) of the crude circular single-stranded DNA were converted into the concatemeric pre-selection libraries with the illustra TempliPhi Amplification Kit (GE Healthcare) according to the manufacturer's protocol. Concatemeric pre-selection libraries were quantified with the Quant-it PicoGreen dsDNA Assay Kit (Invitrogen).

In Vitro Cleavage of on-Target and Off-Target Substrates. Plasmid templates for PCR were constructed by ligation of annealed oligonucleotides CLTA(#) site fwd/rev into HindIII/XbaI double-digested pUC19 (NEB). On-target substrate DNAs were generated by PCR with the plasmid templates and test fwd and test rev primers, then purified with the QIAquick PCR Purification Kit (Qiagen). Off-target substrate DNAs were generated by primer extension. 100 pmol off-target (#) fwd and off-target (#) rev primers were incubated at 95° C. and cooled at 0.1° C./s to 37° C. in NEBuffer2 (50 mM sodium chloride, 10 mM Tris-HCl, 10 mM magnesium chloride, 1 mM dithiothreitol, pH 7.9) supplemented with 10 μM dNTP mix (Bio-Rad). 10 U of Klenow Fragment (3'→5' exo-) (NEB) were added to the reaction mixture and double-stranded off-target templates were obtained by overlap extension for 1 h at 37° C. followed by enzyme inactiva-

tion for 20 min at 75° C., then purified with the QIAquick PCR Purification Kit (Qiagen). 200 nM substrate DNAs were incubated with 100 nM Cas9 and 100 nM (v1.0 or v2.1) sgRNA or 1000 nM Cas9 and 1000 nM (v1.0 or v2.1) sgRNA in Cas9 cleavage buffer (200 mM HEPES, pH 7.5, 1.5 M potassium chloride, 100 mM magnesium chloride, 1 mM EDTA, 5 mM dithiothreitol) for 10 min at 37° C. On-target cleavage reactions were purified with the QIAquick PCR Purification Kit (Qiagen), and off-target cleavage reactions were purified with the QIAquick Nucleotide Removal Kit (Qiagen) before electrophoresis in a Criterion 5% polyacrylamide TBE gel (Bio-Rad).

In Vitro Selection. 200 nM concatemeric pre-selection libraries were incubated with 100 nM Cas9 and 100 nM sgRNA or 1000 nM Cas9 and 1000 nM sgRNA in Cas9 cleavage buffer (200 mM HEPES, pH 7.5, 1.5 M potassium chloride, 100 mM magnesium chloride, 1 mM EDTA, 5 mM dithiothreitol) for 10 min at 37° C. Pre-selection libraries were also separately incubated with 2 U of BspMI restriction endonuclease (NEB) in NEBuffer 3 (100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl2, 1 mM dithiothreitol, pH 7.9) for 1 h at 37° C. Blunt-ended post-selection library members or sticky-ended pre-selection library members were purified with the QIAQuick PCR Purification Kit (Qiagen) and ligated to 10 pmol adapter1/2(AACA) (Cas9:v2.1 sgRNA, 100 nM), adapter1/2(TTCA) (Cas9:v2.1 sgRNA, 1000 nM), adapter1/2 (Cas9:v2.1 sgRNA, 1000 nM), or lib adapter1/CLTA(#) lib adapter 2 (pre-selection) with 1,000 U of T4 DNA Ligase (NEB) in NEB T4 DNA Ligase Reaction Buffer (50 mM Tris-HCl, pH 7.5, 10 mM magnesium chloride, 1 mM ATP, 10 mM dithiothreitol) overnight (>10 h) at room temperature. Adapter-ligated DNA was purified with the QIAquick PCR Purification Kit and PCR-amplified for 10-13 cycles with Phusion Hot Start Flex DNA Polymerase (NEB) in Buffer HF (NEB) and primers CLTA(#) sel PCR/PE2 short (post-selection) or CLTA(#) lib seq PCR/lib fwd PCR (pre-selection). Amplified DNAs were gel purified, quantified with the KAPA Library Quantification Kit-Illumina (KAPA Biosystems), and subjected to single-read sequencing on an Illumina MiSeq or Rapid Run single-read sequencing on an Illumina HiSeq 2500 (Harvard University FAS Center for Systems Biology Core facility, Cambridge, Mass.).

Selection Analysis. Pre-selection and post-selection sequencing data were analyzed as previously described[21], with modification (Algorithms) using scripts written in C++. Raw sequence data is not shown; see Table 2 for a curated summary. Specificity scores were calculated with the formulae: positive specificity score=(frequency of base pair at position[post-selection]−frequency of base pair at position[pre-selection])/(1−frequency of base pair at position[pre-selection]) and negative specificity score=(frequency of base pair at position[post-selection]−frequency of base pair at position[pre-selection])/(frequency of base pair at position [pre-selection]). Normalization for sequence logos was performed as previously described[22].

Cellular Cleavage Assays. HEK293T cells were split at a density of 0.8×10^5 per well (6-well plate) before transcription and maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a 37° C. humidified incubator with 5% CO_2. After 1 day, cells were transiently transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocols. HEK293T cells were transfected at 70% confluency in each well of 6-well plate with 1.0 μg of the Cas9 expression plasmid (Cas9-HA-2×NLS-GFP-NLS) and 2.5 μg of the single-strand RNA expression plasmid pSiliencer-CLTA (version 1.0 or 2.1). The transfection efficiencies were estimated to be ~70%, based on the fraction of GFP-positive cells observed by fluorescence microscopy. 48 h after transfection, cells were washed with phosphate buffered saline (PBS), pelleted and frozen at −80° C. Genomic DNA was isolated from 200 μL cell lysate using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol.

Off-Target Site Sequence Determination. 100 ng genomic DNA isolated from cells treated with Cas9 expression plasmid and single-strand RNA expression plasmid (treated cells) or Cas9 expression plasmid alone (control cells) were amplified by PCR with 10 s 72° C. extension for 35 cycles with primers CLTA(#)-(#)-(#) fwd and CLTA(#)-(#)-(#) rev and Phusion Hot Start Flex DNA Polymerase (NEB) in Buffer GC (NEB), supplemented with 3% DMSO. Relative amounts of crude PCR products were quantified by gel, and Cas9-treated (control) and Cas9:sgRNA-treated PCRs were separately pooled in equimolar concentrations before purification with the QIAquick PCR Purification Kit (Qiagen). Purified DNA was amplified by PCR with primers PE1-barcode# and PE2-barcode# for 7 cycles with Phusion Hot Start Flex DNA Polymerase (NEB) in Buffer HF (NEB). Amplified control and treated DNA pools were purified with the QIAquick PCR Purification Kit (Qiagen), followed by purification with Agencourt AMPure XP (Beckman Coulter). Purified control and treated DNAs were quantified with the KAPA Library Quantification Kit-Illumina (KAPA Biosystems), pooled in a 1:1 ratio, and subjected to paired-end sequencing on an Illumina MiSeq.

Statistical Analysis. Statistical analysis was performed as previously described[21]. P-values in Table 1 and Table 6 were calculated for a one-sided Fisher exact test.

Algorithms

All scripts were written in C++. Algorithms used in this study are as previous reported (reference) with modification.

Sequence binning. 1) designate sequence pairs starting with the barcode "AACA" or "TTCA" as post-selection library members. 2) for post-selection library members (with illustrated example):

Example Read:

(SEQ ID NO: 42)
AACACATGGGTCGACACAAACACAA**CTCGGCAGGT**ACTTGCAGATGTAGT

CTTTCCACATGGGTCGACACAAACACAA**CTCGGCAGGT**ATCTCGTATGCC

i) search both paired reads for the positions, pos1 and pos2, of the constant sequence "CTCGGCAGGT" (SEQ ID NO:43). ii) keep only sequences that have identical sequences between the barcode and pos1 and preceding pos2. iii) keep the region between the two instances of the constant sequence (the region between the barcode and pos1 contains a cut half-site; the region that is between the two instances of the constant sequence contains a full site)

Example:

(SEQ ID NO: 44)
ACTTGCAGATGTAGTCTTTCCACATGGGTCGACACAAACACAA

ii) search the sequence for a selection barcode ("TGTGTTTGTGTT" (SEQ ID NO:45) for CLTA1, "AGAAGAAGAAGA" (SEQ ID NO:46) for CLTA2, "TTCTCTTTCTCT" (SEQ ID NO:47) for CLTA3, "ACACAAACACAA" (SEQ ID NO:48) for CLTA4)

Example:

(SEQ ID NO: 49)
ACTTGCAGATGTAGTCTTTCCACATGGGTCGACACAAACACAA-
CLTA4

iii) the sequence before the barcode is the full post-selection library member (first four and last four nucleotides are fully randomized flanking sequence)

Example:

(SEQ ID NO: 50)
ACTT GCAGATGTAGTCTTTCCACATGG GTCG

iv) parse the quality scores for the positions corresponding to the 23 nucleotide post-selection library member

Example Read:

(SEQ ID NO: 51)
AACACATGGGTCGACACAAACACAACTCGGCAGGTACTTGCAGATGTAGT
CTTTCCACATGGGTCGACACAAACACAACTCGGCAGGTATCTCGTATGCC
CCCFFFFFHHHHHJJJJJJJJJJJJJJJJJJJJGIJJJJIJIJJJIIIH
IIJJJHHHGHAEFCDDDDDDDDDDDDDDDDDDDDD?CDDEDD
@DCCCD

v) keep sequences only if the corresponding quality score string (underlined) FASTQ quality characters for the sequence are '?' or higher in ASCII code (Phred quality score >=30)

NHEJ Sequence Calling

Example Read:

(SEQ ID NO: 52)
CAATCTCCCGCATGCGCTCAGTCCTCATCTCCCTCAAGCAGGCCCCGCTG
GTGCACTGAAGAGCCA**CCCTGTGAAACACTACATCTGC**AATATCTTAATC
CTACTCAGTGAAGCTCTTCACAGTCATTGGATTAATTATGTTGAGTTCTT
TTGGACCAAACC

Example Quality Scores:

CCBCCFFFFCCCGGGGGGGGGGHHHHHHHHHHHHHHHHHHHHGGGGGGGG
GHHHHHHHHHHHHHHH**HHGHHHHHHHHHHHHHHHHHHH**HHGHHHHHHHHH
HHHHHHHHFHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH
HHHGHFHHHHHF

1) identify the 20 base pairs flanking both sides of 20 base pair target site+three base pair PAM for each target site

Example Flanking Sequences:

(SEQ ID NO: 53)
GCTGGTGCACTGAAGAGCCA

(SEQ ID NO: 54)
AATATCTTAATCCTACTCAG

2) search all sequence reads for the flanking sequences to identify the potential off-target site (the sequence between the flanking sequences)

Example Potential Off-target Site:

(SEQ ID NO: 55)
CCCTGTGAAACACTACATCTGC

3) if the potential off-target site contains indels (length is less than 23), keep sequence as potential off-target site if all corresponding FASTQ quality characters for the sequence are '?' or higher in ASCII code (Phred quality score >=30)

Example Potential Off-target Site Length=22

example corresponding FASTQ quality characters: HHGHHHHHHHHHHHHHHHHHHH

4) bin and manually inspect all sequences that pass steps 2 and 3 and keep sequences as potential modified sequences if they have at least one deletion involving position 16, 17, or 18 (of 20 counting from the non-PAM end) of if they have an insertion between position 17 and 18, consistent with the most frequent modifications observed for the intended target site (FIG. **3**)

Example Potential Off-target Site (Reverse Complement, with Positions Labeled) with Reference Sequence:

```
                    11111111112222
non-PAM end 12345678901234567890123 PAM end
```

GCAGATGTAGTGTTTC-ACAGGG (SEQ ID NO: 56)

GCAGATGTAGTGTTTCCACAGGG (SEQ ID NO: 57)

4) repeat steps 1-3 for read2 and keep only if the sequence is the same

5) compare overall counts in Cas9+sgRNA treated sample to Cas9 alone sample to identify modified sites

Filter Based on Cleavage Site (for Post-selection Sequences)

1) tabulate the cleavage site locations across the recognition site by identifying the first position in the full sequenced recognition site (between the two constant sequences) that is identical to the first position in the sequencing read after the barcode (before the first constant sequence).

2) after tabulation, repeat step 1, keeping only sequences with cleavage site locations that are present in at least 5% of the sequencing reads.

Results

Broad Off-target DNA Cleavage Profiling Reveals RNA-programmed Cas9 Nuclease Specificity.

Sequence-specific endonucleases including zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) have become important tools to modify genes in induced pluripotent stem cells (iPSCs),[1-3] in multicellular organisms,[4-8] and in ex vivo gene therapy clinical trials.[9,10] Although ZFNs and TALENs have proved effective for such genetic manipulation, a new ZFN or TALEN protein must be generated for each DNA target site. In contrast, the RNA-guided Cas9 endonuclease uses RNA:DNA hybridization to determine target DNA cleavage sites, enabling a single monomeric protein to cleave, in principle, any sequence specified by the guide RNA.[11]

Previous studies[12-17] demonstrated that Cas9 mediates genome editing at sites complementary to a 20-nucleotide sequence in a bound guide RNA. In addition, target sites must include a protospacer adjacent motif (PAM) at the 3' end adjacent to the 20-nucleotide target site; for *Streptococcus*

pyogenes Cas9, the PAM sequence is NGG. Cas9-mediated DNA cleavage specificity both in vitro and in cells has been inferred previously based on assays against small collections of potential single-mutation off-target sites. These studies suggested that perfect complementarity between guide RNA and target DNA is required in the 7-12 base pairs adjacent to the PAM end of the target site (3' end of the guide RNA) and mismatches are tolerated at the non-PAM end (5' end of the guide RNA).[11, 12, 17-19]

Although such a limited number of nucleotides specifying Cas9:guide RNA target recognition would predict multiple sites of DNA cleavage in genomes of moderate to large size (>~10^7 bp), Cas9:guide RNA complexes have been successfully used to modify both cells[12, 13, 15] and organisms.[14] A study using Cas9:guide RNA complexes to modify zebrafish embryos observed toxicity at a rate similar to that of ZFNs and TALENs.[14] A recent, broad study of the specificity of DNA binding (transcriptional repression) in *E. coli* of a catalytically inactive Cas9 mutant using high-throughput sequencing found no detectable off-target transcriptional repression in the relatively small *E. coli* transcriptome.[20] While these studies have substantially advanced our basic understanding of Cas9, a systematic and comprehensive profile of Cas9:guide RNA-mediated DNA cleavage specificity generated from measurements of Cas9 cleavage on a large number of related mutant target sites has not been described. Such a specificity profile is needed to understand and improve the potential of Cas9:guide RNA complexes as research tools and future therapeutic agents.

We modified our previously published in vitro selection,[21] adapted to process the blunt-ended cleavage products produced by Cas9 compared to the overhang-containing products of ZFN cleavage, to determine the off-target DNA cleavage profiles of Cas9:single guide RNA (sgRNA)[11] complexes. Each selection experiment used DNA substrate libraries containing ~10^{12} sequences, a size sufficiently large to include ten-fold coverage of all sequences with eight or fewer mutations relative to each 22-base pair target sequence (including the two-base pair PAM) (FIG. **1**). We used partially randomized nucleotide mixtures at all 22 target-site base pairs to create a binomially distributed library of mutant target sites with an expected mean of 4.62 mutations per target site. In addition, target site library members were flanked by four fully randomized base pairs on each side to test for specificity patterns beyond those imposed by the canonical 20-base pair target site and PAM.

Pre-selection libraries of 10^{12} individual potential off-target sites were generated for each of four different target sequences in the human clathrin light chain A (CLTA) gene (FIG. **3**). Synthetic 5'-phosphorylated 53-base oligonucleotides were self-ligated into circular single-stranded DNA in vitro, then converted into concatemeric 53-base pair repeats through rolling-circle amplification. The resulting pre-selection libraries were incubated with their corresponding Cas9:sgRNA complexes. Cleaved library members containing free 5' phosphates were separated from intact library members through the 5' phosphate-dependent ligation of non-phosphorylated double-stranded sequencing adapters. The ligation-tagged post-selection libraries were amplified by PCR. The PCR step generated a mixture of post-selection DNA fragments containing 0.5, 1.5, or 2.5, etc. repeats of library members cleaved by Cas9, resulting from amplification of an adapter-ligated cut half-site with or without one or more adjacent corresponding full sites (FIG. **1**). Post-selection library members with 1.5 target-sequence repeats were isolated by gel purification and analyzed by high-throughput sequencing. In a final computational selection step to minimize the impact of errors during DNA amplification or sequencing, only sequences with two identical copies of the repeated cut half-site were analyzed.

Pre-selection libraries were incubated under enzyme-limiting conditions (200 nM target site library, 100 nM Cas9:sgRNA v2.1) or enzyme-saturating conditions (200 nM target site library, 1000 nM Cas9:sgRNA v2.1) for each of the four guide RNAs targets tested (CLTA1, CLTA2, CLTA3, and CLTA4) (FIGS. **3**C and **3**D). A second guide RNA construct, sgRNA v1.0, which is less active than sgRNA v2.1, was assayed under enzyme-saturating conditions alone for each of the four guide RNA targets tested (200 nM target site library, 1000 nM Cas9:sgRNA v1.0). The two guide RNA constructs differ in their length (FIG. **3**) and in their DNA cleavage activity level under the selection conditions, consistent with previous reports[15] (FIG. **4**). Both pre-selection and post-selection libraries were characterized by high-throughput DNA sequencing and computational analysis. As expected, library members with fewer mutations were significantly enriched in post-selection libraries relative to pre-selection libraries (FIG. **5**).

Pre- and Post-Selection Library Composition. The pre-selection libraries for CLTA1, CLTA2, CLTA3, and CLTA4 had observed mean mutation rates of 4.82 (n=1,129,593), 5.06 (n=847,618), 4.66 (n=692,997), and 5.00 (n=951,503) mutations per 22-base pair target site, including the two-base pair PAM, respectively. The post-selection libraries treated under enzyme-limiting conditions with Cas9 plus CLTA1, CLTA2, CLTA3, or CLTA4 v.2.1 sgRNAs contained means of 1.14 (n=1,206,268), 1.21 (n=668,312), 0.91 (n=1,138,568), and 1.82 (n=560,758) mutations per 22-base pair target site. Under enzyme-excess conditions, the mean number of mutations among sequences surviving selection increased to 1.61 (n=640,391), 1.86 (n=399,560), 1.46 (n=936,414), and 2.24 (n=506,179) mutations per 22-base pair target site, respectively, for CLTA1, CLTA2, CLTA3, or CLTA4 v2.1 sgRNAs. These results reveal that the selection significantly enriched library members with fewer mutations for all Cas9:sgRNA complexes tested, and that enzyme-excess conditions resulted in the putative cleavage of more highly mutated library members compared with enzyme-limiting conditions (FIG. **5**).

We calculated specificity scores to quantify the enrichment level of each base pair at each position in the post-selection library relative to the pre-selection library, normalized to the maximum possible enrichment of that base pair. Positive specificity scores indicate base pairs that were enriched in the post-selection library and negative specificity scores indicate base pairs that were de-enriched in the post-selection library. For example, a score of +0.5 indicates that a base pair is enriched to 50% of the maximum enrichment value, while a score of −0.5 indicates that a base pair is de-enriched to 50% of the maximum de-enrichment value.

In addition to the two base pairs specified by the PAM, all 20 base pairs targeted by the guide RNA were enriched in the sequences from the CLTA1 and CLTA2 selections (FIG. **2**, FIGS. **6** and **9**, and Table 2). For the CLTA3 and CLTA4 selections (FIGS. **7** and **8**, and Table 2), guide RNA-specified base pairs were enriched at all positions except for the two most distal base pairs from the PAM (5' end of the guide RNA), respectively. At these non-specified positions farthest from the PAM, at least two of the three alternate base pairs were nearly as enriched as the specified base pair. Our finding that the entire 20 base-pair target site and two base pair PAM can contribute to Cas9:sgRNA DNA cleavage specificity con-

trasts with the results from previous single-substrate assays suggesting that only 7-12 base pairs and two base pair PAM are specified.[11, 12, 15]

All single-mutant pre-selection (n≥14,569) and post-selection library members (n≥103,660) were computationally analyzed to provide a selection enrichment value for every possible single-mutant sequence. The results of this analysis (FIG. **2** and FIGS. **6** and **8**) show that when only single-mutant sequences are considered, the six to eight base pairs closest to the PAM are generally highly specified and the non-PAM end is poorly specified under enzyme-limiting conditions, consistent with previous findings.[11, 12, 17-19] Under enzyme-saturating conditions, however, single mutations even in the six to eight base pairs most proximal to the PAM are tolerated, suggesting that the high specificity at the PAM end of the DNA target site can be compromised when enzyme concentrations are high relative to substrate (FIG. **2**). The observation of high specificity against single mutations close to the PAM only applies to sequences with a single mutation and the selection results do not support a model in which any combination of mutations is tolerated in the region of the target site farthest from the PAM (FIG. **10-15**). Analyses of pre- and post-selection library composition are described elsewhere herein, position-dependent specificity patterns are illustrated in FIGS. **18-20**, PAM nucleotide specificity is illustrated in FIGS. **21-24**, and more detailed effects of Cas9:sgRNA concentration on specificity are described in FIG. **2**G and FIG. **25**).

Specificity at the Non-PAM End of the Target Site. To assess the ability of Cas9:v2.1 sgRNA under enzyme-excess conditions to tolerate multiple mutations distal to the PAM, we calculated maximum specificity scores at each position for sequences that contained mutations only in the region of one to 12 base pairs at the end of the target site most distal from the PAM (FIG. **10-17**).

The results of this analysis show no selection (maximum specificity score ~0) against sequences with up to three mutations, depending on the target site, at the end of the molecule farthest from the PAM when the rest of the sequence contains no mutations. For example, when only the three base pairs farthest from the PAM are allowed to vary (indicated by dark bars in FIG. **11**C) in the CLTA2 target site, the maximum specificity scores at each of the three variable positions are close to zero, indicating that there was no selection for any of the four possible base pairs at each of the three variable positions. However, when the eight base pairs farthest from the PAM are allowed to vary (FIG. **11**H), the maximum specificity scores at positions 4-8 are all greater than +0.4, indicating that the Cas9:sgRNA has a sequence preference at these positions even when the rest of the substrate contains preferred, on-target base pairs.

We also calculated the distribution of mutations (FIG. **15-17**), in both pre-selection and v2.1 sgRNA-treated post-selection libraries under enzyme-excess conditions, when only the first 1-12 base pairs of the target site are allowed to vary. There is significant overlap between the pre-selection and post-selection libraries for only a subset of the data (FIG. **15-17**, *a-c*), demonstrating minimal to no selection in the post-selection library for sequences with mutations only in the first three base pairs of the target site. These results collectively show that Cas9:sgRNA can tolerate a small number of mutations (~one to three) at the end of the sequence farthest from the PAM when provided with maximal sgRNA:DNA interactions in the rest of the target site.

Specificity at the PAM End of the Target Site. We plotted positional specificity as the sum of the magnitudes of the specificity scores for all four base pairs at each position of each target site, normalized to the same sum for the most highly specified position (FIG. **18-20**). Under both enzyme-limiting and enzyme-excess conditions, the PAM end of the target site is highly specified. Under enzyme-limiting conditions, the PAM end of the molecule is almost absolutely specified (specificity score ≥+0.9 for guide RNA-specified base pairs) by CLTA1, CTLA2, and CLTA3 guide RNAs (FIG. **2** and FIG. **6-9**), and highly specified by CLTA4 guide RNA (specificity score of +0.7 to +0.9). Within this region of high specificity, specific single mutations, consistent with wobble pairing between the guide RNA and target DNA, that are tolerated. For example, under enzyme-limiting conditions for single-mutant sequences, a dA:dT off-target base pair and a guide RNA-specified dG:dC base pair are equally tolerated at position 17 out of 20 (relative to the non-PAM end of the target site) of the CLTA3 target site. At this position, an rG:dT wobble RNA:DNA base pair may be formed, with minimal apparent loss of cleavage activity.

Importantly, the selection results also reveal that the choice of guide RNA hairpin affects specificity. The shorter, less-active sgRNA v1.0 constructs are more specific than the longer, more-active sgRNA v2.1 constructs when assayed under identical, enzyme-saturating conditions that reflect an excess of enzyme relative to substrate in a cellular context (FIG. **2** and FIGS. **5-8**). The higher specificity of sgRNA v1.0 over sgRNA v2.1 is greater for CLTA1 and CLTA2 (~40-90% difference) than for CLTA3 and CLTA4 (<40% difference). Interestingly, this specificity difference is localized to different regions of the target site for each target sequence (FIGS. **2**H and **26**). Collectively, these results indicate that different guide RNA architectures result in different DNA cleavage specificities, and that guide RNA-dependent changes in specificity do not affect all positions in the target site equally. Given the inverse relationship between Cas9:sgRNA concentration and specificity described above, we speculate that the differences in specificity between guide RNA architectures arises from differences in their overall level of DNA-cleavage activities.

Effects of Cas9:sgRNA Concentration on DNA Cleavage Specificity. To assess the effect of enzyme concentration on patterns of specificity for the four target sites tested, we calculated the concentration-dependent difference in positional specificity and compared it to the maximal possible change in positional specificity (FIG. **25**). In general, specificity was higher under enzyme-limiting conditions than enzyme-excess conditions. A change from enzyme-excess to enzyme-limiting conditions generally increased the specificity at the PAM end of the target by ≥80% of the maximum possible change in specificity. Although a decrease in enzyme concentration generally induces small (~30%) increases in specificity at the end of the target sites farthest from the PAM, concentration decreases induce much larger increases in specificity at the end of the target site nearest the PAM. For CLTA4, a decrease in enzyme concentration is accompanied by a small (~30%) decrease in specificity at some base pairs near the end of the target site farthest from the PAM.

Specificity of PAM Nucleotides. To assess the contribution of the PAM to specificity, we calculated the abundance of all 16 possible PAM dinucleotides in the pre-selection and post-selection libraries, considering all observed post-selection target site sequences (FIG. **21**) or considering only post-selection target site sequences that contained no mutations in the 20 base pairs specified by the guide RNA (FIG. **22**). Considering all observed post-selection target site sequences, under enzyme-limiting conditions, GG dinucleotides represented 99.8%, 99.9%, 99.8%, and 98.5% of the post-selection PAM dinucleotides for selections with CLTA1, CLTA2,

CLTA3, and CLTA4 v2.1 sgRNAs, respectively. In contrast, under enzyme-excess conditions, GG dinucleotides represented 97.7%, 98.3%, 95.7%, and 87.0% of the post-selection PAM dinucleotides for selections with CLTA1, CLTA2, CLTA3, and CLTA4 v2.1 sgRNAs, respectively. These data demonstrate that an increase in enzyme concentration leads to increased cleavage of substrates containing non-canonical PAM dinucleotides.

To account for the pre-selection library distribution of PAM dinucleotides, we calculated specificity scores for the PAM dinucleotides (FIG. **23**). When only on-target post-selection sequences are considered under enzyme-excess conditions (FIG. **24**), non-canonical PAM dinucleotides with a single G rather than two Gs are relatively tolerated. Under enzyme-excess conditions, Cas9:CLTA4 sgRNA 2.1 exhibited the highest tolerance of non-canonical PAM dinucleotides of all the Cas9:sgRNA combinations tested. AG and GA dinucleotides were the most tolerated, followed by GT, TG, and CG PAM dinucleotides. In selections with Cas9:CLTA1, 2, or 3 sgRNA 2.1 under enzyme-excess conditions, AG was the predominate non-canonical PAM (FIGS. **23** and **24**). Our results are consistent with another recent study of PAM specificity, which shows that Cas9:sgRNA can recognize AG PAM dinucleotides[23]. In addition, our results show that under enzyme-limiting conditions, GG PAM dinucleotides are highly specified, and under enzyme-excess conditions, non-canonical PAM dinucleotides containing a single G can be tolerated, depending on the guide RNA context.

To confirm that the in vitro selection results accurately reflect the cleavage behavior of Cas9 in vitro, we performed discrete cleavage assays of six CLTA4 off-target substrates containing one to three mutations in the target site. We calculated enrichment values for all sequences in the post-selection libraries for the Cas9:CLTA4 v2.1 sgRNA under enzyme-saturating conditions by dividing the abundance of each sequence in the post-selection library by the calculated abundance in the pre-selection library. Under enzyme-saturating conditions, the single one, two, and three mutation sequences with the highest enrichment values (27.5, 43.9, and 95.9) were cleaved to ≥71% completion (FIG. **27**). A two-mutation sequence with an enrichment value of 1.0 was cleaved to 35%, and a two-mutation sequence with an enrichment value near zero (0.064) was not cleaved. The three-mutation sequence, which was cleaved to 77% by CLTA4 v2.1 sgRNA, was cleaved to a lower efficiency of 53% by CLTA4 v1.0 sgRNA (FIG. **28**). These results indicate that the selection enrichment values of individual sequences are predictive of in vitro cleavage efficiencies.

To determine if results of the in vitro selection and in vitro cleavage assays pertain to Cas9:guide RNA activity in human cells, we identified 51 off-target sites (19 for CLTA1 and 32 for CLTA4) containing up to eight mutations that were both enriched in the in vitro selection and present in the human genome (Tables 3-5). We expressed Cas9:CLTA1 sgRNA v1.0, Cas9:CLTA1 sgRNA v2.1, Cas9:CLTA4 sgRNA v1.0, Cas9:CLTA4 sgRNA v2.1, or Cas9 without sgRNA in HEK293T cells by transient transfection and used genomic PCR and high-throughput DNA sequencing to look for evidence of Cas9:sgRNA modification at 46 of the 51 off-target sites as well as at the on-target loci; no specific amplified DNA was obtained for five of the 51 predicted off-target sites (three for CLTA1 and two for CLTA4).

Deep sequencing of genomic DNA isolated from HEK293T cells treated with Cas9:CLTA1 sgRNA or Cas9:CLTA4 sgRNA identified sequences evident of non-homologous end-joining (NHEJ) at the on-target sites and at five of the 49 tested off-target sites (CLTA1-1-1, CLTA1-2-2, CLTA4-3-1, CLTA4-3-3, and CLTA4-4-8) (Tables 1 and 6-8). The CLTA4 target site was modified by Cas9:CLTA4 v2.1 sgRNA at a frequency of 76%, while off-target sites, CLTA4-3-1 CLTA4-3-3, and CLTA4-4-8, were modified at frequencies of 24%, 0.47% and 0.73%, respectively. The CLTA1 target site was modified by Cas9:CLTA1 v2.1 sgRNA at a frequency of 0.34%, while off-target sites, CLTA1-1-1 and CLTA1-2-2, were modified at frequencies of 0.09% and 0.16%, respectively.

Under enzyme-saturating conditions with the v2.1 sgRNA, the two verified CLTA1 off-target sites, CLTA1-1-1 and CLTA1-2-2, were two of the three most highly enriched sequences identified in the in vitro selection. CLTA4-3-1 and CLTA4-3-3 were the highest and third-highest enriched sequences of the seven CLTA4 three-mutation sequences enriched in the in vitro selection that are also present in the genome. The in vitro selection enrichment values of the four-mutation sequences were not calculated, since 12 out of the 14 CLTA4 sequences in the genome containing four mutations, including CLTA4-4-8, were observed at a level of only one sequence count in the post-selection library. Taken together, these results confirm that several of the off-target substrates identified in the in vitro selection that are present in the human genome are indeed cleaved by Cas9:sgRNA complexes in human cells, and also suggest that the most highly enriched genomic off-target sequences in the selection are modified in cells to the greatest extent.

The off-target sites we identified in cells were among the most-highly enriched in our in vitro selection and contain up to four mutations relative to the intended target sites. While it is possible that heterochromatin or covalent DNA modifications could diminish the ability of a Cas9:guide RNA complex to access genomic off-target sites in cells, the identification of five out of 49 tested cellular off-target sites in this study, rather than zero or many, strongly suggests that Cas9-mediated DNA cleavage is not limited to specific targeting of only a 7-12-base pair target sequence, as suggested in recent studies.[11, 12, 19]

The cellular genome modification data are also consistent with the increase in specificity of sgRNA v1.0 compared to sgRNA v2.1 sgRNAs observed in the in vitro selection data and discrete assays. Although the CLTA1-2-2, CLTA 4-3-3, and CLTA 4-4-8 sites were modified by the Cas9-sgRNA v2.1 complexes, no evidence of modification at any of these three sites was detected in Cas9:sgRNA v1.0-treated cells. The CLTA4-3-1 site, which was modified at 32% of the frequency of on-target CLTA4 site modification in Cas9:v2.1 sgRNA-treated cells, was modified at only 0.5% of the on-target modification frequency in v1.0 sgRNA-treated cells, representing a 62-fold change in selectivity. Taken together, these results demonstrate that guide RNA architecture can have a significant influence on Cas9 specificity in cells. Our specificity profiling findings present an important caveat to recent and ongoing efforts to improve the overall DNA modification activity of Cas9:guide RNA complexes through guide RNA engineering.[11, 15]

Overall, the off-target DNA cleavage profiling of Cas9 and subsequent analyses show that (i) Cas9:guide RNA recognition extends to 18-20 specified target site base pairs and a two-base pair PAM for the four target sites tested; (ii) increasing Cas9:guide RNA concentrations can decrease DNA-cleaving specificity in vitro; (iii) using more active sgRNA architectures can increase DNA-cleavage specificity both in vitro and in cells but impair DNA-cleavage specificity both in vitro and in cells; and (iv) as predicted by our in vitro results, Cas9:guide RNA can modify off-target sites in cells with up to four mutations relative to the on-target site. Our findings

provide key insights to our understanding of RNA-programmed Cas9 specificity, and reveal a previously unknown role for sgRNA architecture in DNA-cleavage specificity. The principles revealed in this study may also apply to Cas9-based effectors engineered to mediate functions beyond DNA cleavage.

Equivalents and Scope

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. The scope of the present invention is not intended to be limited to the above description, but rather is as set forth in the appended claims.

In the claims articles such as "a," "an," and "the" may mean one or more than one unless indicated to the contrary or otherwise evident from the context. Claims or descriptions that include "or" between one or more members of a group are considered satisfied if one, more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process unless indicated to the contrary or otherwise evident from the context. The invention includes embodiments in which exactly one member of the group is present in, employed in, or otherwise relevant to a given product or process. The invention also includes embodiments in which more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process.

Furthermore, it is to be understood that the invention encompasses all variations, combinations, and permutations in which one or more limitations, elements, clauses, descriptive terms, etc., from one or more of the claims or from relevant portions of the description is introduced into another claim. For example, any claim that is dependent on another claim can be modified to include one or more limitations found in any other claim that is dependent on the same base claim. Furthermore, where the claims recite a composition, it is to be understood that methods of using the composition for any of the purposes disclosed herein are included, and methods of making the composition according to any of the methods of making disclosed herein or other methods known in the art are included, unless otherwise indicated or unless it would be evident to one of ordinary skill in the art that a contradiction or inconsistency would arise.

Where elements are presented as lists, e.g., in Markush group format, it is to be understood that each subgroup of the elements is also disclosed, and any element(s) can be removed from the group. It is also noted that the term "comprising" is intended to be open and permits the inclusion of additional elements or steps. It should be understood that, in general, where the invention, or aspects of the invention, is/are referred to as comprising particular elements, features, steps, etc., certain embodiments of the invention or aspects of the invention consist, or consist essentially of, such elements, features, steps, etc. For purposes of simplicity those embodiments have not been specifically set forth in haec verba herein. Thus for each embodiment of the invention that comprises one or more elements, features, steps, etc., the invention also provides embodiments that consist or consist essentially of those elements, features, steps, etc.

Where ranges are given, endpoints are included. Furthermore, it is to be understood that unless otherwise indicated or otherwise evident from the context and/or the understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value within the stated ranges in different embodiments of the invention, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise. It is also to be understood that unless otherwise indicated or otherwise evident from the context and/or the understanding of one of ordinary skill in the art, values expressed as ranges can assume any subrange within the given range, wherein the endpoints of the subrange are expressed to the same degree of accuracy as the tenth of the unit of the lower limit of the range.

In addition, it is to be understood that any particular embodiment of the present invention may be explicitly excluded from any one or more of the claims. Where ranges are given, any value within the range may explicitly be excluded from any one or more of the claims. Any embodiment, element, feature, application, or aspect of the compositions and/or methods of the invention, can be excluded from any one or more claims. For purposes of brevity, all of the embodiments in which one or more elements, features, purposes, or aspects is excluded are not set forth explicitly herein.

Tables

Table 1. Cellular modification induced by Cas9:CLTA4 sgRNA.

33 human genomic DNA sequences were identified that were enriched in the Cas9:CLTA4 v2.1 sgRNA in vitro selections under enzyme-limiting or enzyme-saturating conditions. Sites shown with underline contain insertions or deletions (indels) that are consistent with significant Cas9:sgRNA-mediated modification in HEK293T cells. In vitro enrichment values for selections with Cas9:CLTA4 v1.0 sgRNA or Cas9:CLTA4 v2.1 sgRNA are shown for sequences with three or fewer mutations. Enrichment values were not calculated for sequences with four or more mutations due to low numbers of in vitro selection sequence counts. Modification frequencies (number of sequences with indels divided by total number of sequences) in HEK293T cells treated with Cas9 without sgRNA ("no sgRNA"), Cas9 with CLTA4 v1.0 sgRNA, or Cas9 with CLTA4 v2.1 sgRNA. P-values are listed for those sites that show significant modification in v1.0 sgRNA- or v2.1 sgRNA-treated cells compared to cells treated with Cas9 without sgRNA. "Not tested (n.t.)" indicates that PCR of the genomic sequence failed to provide specific amplification products.

Table 2: Raw selection sequence counts. Positions −4 to −1 are the four nucleotides preceding the 20-base pair target site. PAM1, PAM2, and PAM3 are the PAM positions immediately following the target site. Positions+4 to +7 are the four nucleotides immediately following the PAM.

Table 3: CLTA1 genomic off-target sequences. 20 human genomic DNA sequences were identified that were enriched in the Cas9:CLTA1 v2.1 sgRNA in vitro selections under enzyme-limiting or enzyme-excess conditions. "m" refers to number of mutations from on-target sequence with mutations shown in lower case. Sites shown with underline contain insertions or deletions (indels) that are consistent with significant Cas9:sgRNA-mediated modification in HEK293T cells. Human genome coordinates are shown for each site (assembly GRCh37). CLTA1-0-1 is present at two loci, and sequence counts were pooled from both loci. Sequence counts are shown for amplified and sequenced DNA for each site from HEK293T cells treated with Cas9 without sgRNA ("no sgRNA"), Cas9 with CLTA1 v1.0 sgRNA, or Cas9 with CLTA1 v2.1 sgRNA.

Table 4: CLTA4 genomic off-target sequences. 33 human genomic DNA sequences were identified that were enriched in the Cas9:CLTA4 v2.1 sgRNA in vitro selections under

enzyme-limiting or enzyme-excess conditions. "m" refers to number of mutations from on-target sequence with mutations shown in lower case. Sites shown with underline contain insertions or deletions (indels) that are consistent with significant Cas9:sgRNA-mediated modification in HEK293T cells. Human genome coordinates are shown for each site (assembly GRCh37). Sequence counts are shown for amplified and sequenced DNA for each site from HEK293T cells treated with Cas9 without sgRNA ("no sgRNA"), Cas9 with CLTA4 v1.0 sgRNA, or Cas9 with CLTA4 v2.1 sgRNA.

Table 5: genomic coordinates of CLTA1 and CLTA4 off-target sites. 54 human genomic DNA sequences were identified that were enriched in the Cas9:CLTA1 v2.1 sgRNA and Cas9:CLTA4 v2.1 sgRNA in vitro selections under enzyme-limiting or enzyme-excess conditions. Human genome coordinates are shown for each site (assembly GRCh37).

Table 6: Cellular modification induced by Cas9:CLTA1 sgRNA. 20 human genomic DNA sequences were identified that were enriched in the Cas9:CLTA1 v2.1 sgRNA in vitro selections under enzyme-limiting or enzyme-excess conditions. Sites shown with underline contain insertions or deletions (indels) that are consistent with significant Cas9:sgRNA-mediated modification in HEK293T cells. In vitro enrichment values for selections with Cas9:CLTA1 v1.0 sgRNA or Cas9:CLTA1 v2.1 sgRNA are shown for sequences with three or fewer mutations. Enrichment values were not calculated for sequences with four or more mutations due to low numbers of in vitro selection sequence counts. Modification frequencies (number of sequences with indels divided by total number of sequences) in HEK293T cells treated with Cas9 without sgRNA ("no sgRNA"), Cas9 with CLTA1 v1.0 sgRNA, or Cas9 with CLTA1 v2.1 sgRNA. P-values of sites that show significant modification in v1.0 sgRNA- or v2.1 sgRNA-treated cells compared to cells treated with Cas9 without sgRNA were 1.1E-05 (v1.0) and 6.9E-55 (v2.1) for CLTA1-0-1, 2.6E-03 (v1.0) and 2.0E-10 (v2.1) for CLTA1-1-1, and 4.6E-08 (v2.1) for CLTA1-2-2. P-values were calculated using a one-sided Fisher exact test. "Not tested (n.t.)" indicates that the site was not tested or PCR of the genomic sequence failed to provide specific amplification products.

Table 7: CLTA1 genomic off-target indel sequences. Insertion and deletion-containing sequences from cells treated with amplified and sequenced DNA for the on-target genomic sequence (CLTA1-0-1) and each modified off-target site from HEK293T cells treated with Cas9 without sgRNA ("no sgRNA"), Cas9 with CLTA1 v1.0 sgRNA, or Cas9 with CLTA1 v2.1 sgRNA. "ref" refers to the human genome reference sequence for each site, and the modified sites are listed below. Mutations relative to the on-target genomic sequence are shown in lowercase letters. Insertions and deletions are shown in underlined bold letters or dashes, respectively. Modification percentages are shown for those conditions (v1.0 sgRNA or v2.1 sgRNA) that show statistically significant enrichment of modified sequences compared to the control (no sgRNA).

Table 8: CLTA4 genomic off-target indel sequences. Insertion and deletion-containing sequences from cells treated with amplified and sequenced DNA for the on-target genomic sequence (CLTA4-0-1) and each modified off-target site from HEK293T cells treated with Cas9 without sgRNA ("no sgRNA"), Cas9 with CLTA4 v1.0 sgRNA, or Cas9 with CLTA4 v2.1 sgRNA. "ref" refers to the human genome reference sequence for each site, and the modified sites are listed below. Mutations relative to the on-target genomic sequence are shown in lowercase letters. Insertions and deletions are shown in underlined bold letters or dashes, respectively. Modification percentages are shown for those conditions (v1.0 sgRNA or v2.1 sgRNA) that show statistically significant enrichment of modified sequences compared to the control (no sgRNA).

Table 9: Oligonucleotides used in this study. All oligonucleotides were purchased from Integrated DNA Technologies. An asterisk (*) indicates that the preceding nucleotide was incorporated as a hand mix of phosphoramidites consisting of 79 mol % of the phosphoramidite corresponding to the preceding nucleotide and 4 mol % of each of the other three canonical phosphoramidites. "/SPhos/" denotes a 5' phosphate group installed during synthesis.

TABLE 1

	# of Mutations	sequence	SEQ ID NO.	gene	in vitro enrichment v1.0	in vitro enrichment v2.1
CLTA4-0-1	0	GCAGATGTAGTGTTTCCACAGGG	SEQ ID NO: 58	CLTA	20	7.95
CLTA4-3-1	3	aCAtATGTAGTaTTTCCACAGGG	SEQ ID NO: 59		16.5	12.5
CLTA4-3-2	3	GCAtATGTAGTGTTTCCAaATGt	SEQ ID NO: 60		2.99	6.97
CLTA4-3-3	3	cCAGATGTAGTaTTcCCACAGGG	SEQ ID NO: 61	CELF1	1.00	4.95
CLTA4-3-4	3	GCAGtTtTAGTGTTTtCACAGGG	SEQ ID NO: 62	BC073807	0.79	3.12
CLTA4-3-5	3	GCAGAgtTAGTGTTTCCACACaG	SEQ ID NO: 63	MPPED2	0	1.22
CLTA4-3-6	3	GCAGATGgAGgGTTTtCACAGGG	SEQ ID NO: 64	DCHS2	1.57	1.17
CLTA4-3-7	3	GgAaATtTAGTGTTTCCACAGGG	SEQ ID NO: 65		0.43	0.42
CLTA4-4-1	4	aaAGAaGTAGTaTTTCCACATGG	SEQ ID NO: 66			
CLTA4-4-2	4	aaAGATGTAGTcaTTCCACAAGG	SEQ ID NO: 67			
CLTA4-4-3	4	aaAtATGTAGTcTTTCCACAGGG	SEQ ID NO: 68			
CLTA4-4-4	4	atAGATGTAGTGTTTCCAaAGGa	SEQ ID NO: 69	NR1H4		
CLTA4-4-5	4	cCAGAgGTAGTGcTcCCACAGGG	SEQ ID NO: 70			

TABLE 1-continued

CLTA4-4-6	4	cCAGATGTgagGTTTCCACAAGG	SEQ ID NO: 71	XKR6	
CLTA4-4-7	4	ctAcATGTAGTGTTTCCAtATGG	SEQ ID NO: 72	HKR1	
CLTA4-4-8	4	ctAGATGaAGTGcTTCCACATGG	SEQ ID NO: 73	CDK8	
CLTA4-4-9	4	GaAaATGgAGTGTTTaCACATGG	SEQ ID NO: 74		
CLTA4-4-10	4	GCAaATGaAGTGTcaCCACAAGG	SEQ ID NO: 75		
CLTA4-4-11	4	GCAaATGTAtTaTTTCCACtAGG	SEQ ID NO: 76	NOV	
CLTA4-4-12	4	GCAGATGTAGctTTTgtACATGG	SEQ ID NO: 77		
CLTA4-4-13	4	GCAGcTtaAGTGTTTtCACATGG	SEQ ID NO: 78	GRHL2	
CLTA4-4-14	4	ttAcATGTAGTGTTTaCACACGG	SEQ ID NO: 79	LINC00535	
CLTA4-5-1	5	GaAGAGgAAGTGTTTgCcCAGGG	SEQ ID NO: 80	RNH1	
CLTA4-5-2	5	GaAGATGTgGaGTTgaCACATGG	SEQ ID NO: 81	FZD3	
CLTA4-5-3	5	GCAGAaGTAcTGTTgttACAAGG	SEQ ID NO: 82		
CLTA4-5-4	5	GCAGATGTgGaaTTaCaACAGGG	SEQ ID NO: 83	SLC9A2	
CLTA4-5-5	5	GCAGtcaTAGTGTaTaCACATGG	SEQ ID NO: 84		
CLTA4-5-6	5	taAGATGTAGTaTTTCCAaAAGt	SEQ ID NO: 85		
CLTA4-6-1	6	GCAGcTGgcaTtTcTCCACACGG	SEQ ID NO: 86		
CLTA4-6-2	6	GgAGATcTgaTGgTTCtACAAGG	SEQ ID NO: 87		
CLTA4-6-3	6	taAaATGcAGTGTaTCCAtATGG	SEQ ID NO: 88	SMA4	
CLTA4-7-1	7	GCcagaaTAGTtTTTCaACAAGG	SEQ ID NO: 89	SEPHS2	
CLTA4-7-2	8	ttgtATtTAGaGaTTgCACAAGG	SEQ ID NO: 90	RORB	

	modification frequency in HEK293T cells			P-value	
	no sgRNA	v1.0	v2.1	v1.0	v2.1
CLTA4-0-1	0.021%	11%	76%	<1E-55	<1E-55
CLTA4-3-1	0.006%	0.055%	24%	6.0E-04	<1E-55
CLTA4-3-2	0.017%	0%	0.014%		
CLTA4-3-3	0%	0%	0.469%		2.5E-21
CLTA4-3-4	0%	0%	0%		
CLTA4-3-5	0.005%	0.015%	0.018%		
CLTA4-3-6	0.015%	0.023%	0.021%		
CLTA4-3-7	0.005%	0.012%	0.003%		
CLTA4-4-1	n.t.	n.t.	n.t.		
CLTA4-4-2	0.004%	0%	0.005%		
CLTA4-4-3	0.004%	0.009%	0%		
CLTA4-4-4	0.032%	0.006%	0.052%		
CLTA4-4-5	0.005%	0.006%	0.007%		
CLTA4-4-6	0.018%	0%	0.007%		
CLTA4-4-7	0.006%	0%	0.008%		

TABLE 1-continued

CLTA4-4-8	0.009%	0.013%	0.730%	9.70E-21
CLTA4-4-9	0%	0%	0.004%	
CLTA4-4-10	0.004%	0%	0%	
CLTA4-4-11	0%	0.00%	0%	
CLTA4-4-12	0%	0.00%	0%	
CLTA4-4-13	0.020%	0.02%	0.030%	
CLTA4-4-14	n.t.	n.t.	n.t.	
CLTA4-5-1	0.004%	0.01%	0.006%	
CLTA4-5-2	0.004%	0.00%	0%	
CLTA4-5-3	0.002%	0.00%	0.003%	
CLTA4-5-4	0%	0.00%	0%	
CLTA4-5-5	0.004%	0.00%	0.005%	
CLTA4-5-6	0.007%	0.01%	0%	
CLTA4-6-1	n.t.	n.t.	n.t.	
CLTA4-6-2	0.007%	0.00%	0.009%	
CLTA4-6-3	0.015%	0.00%	0%	
CLTA4-7-1	0%	0.00%	0.007%	
CLTA4-7-2	0%	0.00%	0%	

TABLE 2

100 nM Cas9:CLTA1 v2.1 sgRNA

position	−4	−3	−2	−1	1	2	3	4	5	6	7	8	9	10	11	12
A	212906	240335	195549	240068	1.04E+06	72751	40206	62972	41734	17376	18710	1.17E+06	24455	83195	46083	33528
C	285295	248395	263973	260202	37925	32496	24822	1.10E+06	1.12E+06	42444	1.16E+06	5339	22096	1.06E+06	48105	1.14E+06
G	214213	219078	220275	189578	61062	1.04E+06	25785	11117	9125	5423	5745	5121	8080	14905	8906	3732
T	493854	498460	526471	516420	64694	59173	1.12E+06	35336	34236	1.14E+06	20532	24018	1.15E+06	50488	1.10E+06	32417

1000 nM Cas9:CLTA1 v1.0 sgRNA

position	−4	−3	−2	−1	1	2	3	4	5	6	7	8	9	10	11	12
A	154613	184336	154288	177436	805105	66777	43354	56461	32941	15531	19465	904223	19696	56566	35200	26674
C	227144	201856	215667	220894	30269	30133	24249	825333	865486	35164	889622	5488	17340	828521	36975	876790
G	163868	174062	177891	148150	47940	784264	26342	17972	10299	6332	5785	5938	9185	11560	10641	3020
T	389059	374430	386838	388204	51370	53510	840739	34918	25958	877657	19812	19035	888463	38037	851868	28200

1000 nM Cas9:CLTA1 v2.1 sgRNA

position	−4	−3	−2	−1	1	2	3	4	5	6	7	8	9	10	11	12
A	104782	127116	103361	124521	554601	40232	29541	38710	23659	10435	11462	618404	14608	41826	27762	19590
C	154144	136337	145670	146754	20057	19440	17922	569754	590426	25233	612203	3834	15297	561351	26392	592757
G	113998	119668	120741	103026	32861	547445	18468	9314	6346	3908	4295	3719	5851	10887	15360	5605
T	267467	257270	270619	266090	32872	33274	574460	22613	19960	600815	12431	14434	604635	26327	570877	22439

CLTA1 pre-selection library

position	−4	−3	−2	−1	1	2	3	4	5	6	7	8	9	10	11	12
A	241543	217144	209045	198284	943175	103452	76259	106919	124476	59762	108373	937511	65477	110282	67774	96299
C	254366	269805	276090	322860	52984	65855	58943	834238	812029	52168	839963	54708	43285	831610	50109	861358
G	230024	196574	210445	180859	60496	857631	66783	89366	85315	67098	77499	59257	71824	89579	68090	66121
T	403590	446000	433943	427520	72868	102585	927538	99000	107703	950495	103688	78047	948937	98052	943550	105745

100 nM Cas9:CLTA2 v2.1 sgRNA

position	−4	−3	−2	−1	1	2	3	4	5	6	7	8	9	10	11	12
A	109129	135587	94032	141748	5.74E+04	44802	48284	24464	11611	16668	6282	6.58E+05	655917	28909	24210	656617
C	155710	138970	207735	220443	529643	24503	566049	6.27E+05	6.46E+05	19040	6.52E+05	2351	2577	1.30E+04	617274	2.64E+03
G	136555	142038	118241	105620	39991	2.11E+04	26481	3756	3627	2889	2488	3025	3202	609865	8312	5889
T	266918	251717	248304	200501	41277	577893	2.75E+04	13008	7318	6.30E+05	7487	4920	6.62E+03	16554	1.85E+04	3165

1000 nM Cas9:CLTA2 v1.0 sgRNA

position	−4	−3	−2	−1	1	2	3	4	5	6	7	8	9	10	11	12
A	94138	115628	85485	120676	52411	41438	46093	22399	9066	14310	5351	567337	565061	24132	23848	556483
C	140695	125708	179224	191394	452192	21517	481298	538392	557549	16233	562576	1973	2127	11807	525901	4992

TABLE 2-continued

G	113243	118054	101836	91048	35101	18969	22797	3440	2802	2960	2526	2895	2793	526655	9738	8100
T	228367	217053	209898	173125	36739	494519	26255	12212	7026	542940	5990	4238	6462	13849	16956	4868
1000 nM Cas9:CLTA2 v2.1 sgRNA																
position	−4	−3	−2	−1	1	2	3	4	5	6	7	8	9	10	11	12
A	64249	81812	58977	85387	35172	29833	33434	19419	9272	13136	4907	391675	389930	19852	16657	383605
C	96983	67918	124642	127760	316077	14548	327166	364874	380987	11360	387025	1694	1815	8124	363374	5168
G	77913	80500	68612	64299	23522	15748	19664	3856	3035	2752	2062	2398	2439	360755	7431	6019
T	160415	149330	147329	122114	24789	339431	19296	11411	6266	372312	5566	3793	5376	10829	12098	4768
CLTA2 pre-selection library																
position	−4	−3	−2	−1	1	2	3	4	5	6	7	8	9	10	11	12
A	203147	173899	167999	170203	89970	73770	88239	88611	76114	78589	75016	726091	712150	96111	90307	728931
C	181430	214835	246369	272618	632831	41977	641062	644565	670872	40877	649838	38931	44961	46591	628706	32296
G	177090	153006	151178	140868	58664	49976	60827	56077	52341	49259	55484	39801	38939	630670	55013	38368
T	285951	305878	282072	263929	66153	681895	57490	58365	48291	678893	67280	42795	51838	74246	73592	48023
100 nM Cas9:CLT3A v2.1 sgRNA																
position	−4	−3	−2	−1	1	2	3	4	5	6	7	8	9	10	11	12
A	212836	248582	202151	249368	9.13E+04	77392	19048	39738	1078520	1106930	46196	1.12E+06	64461	11912	30992	21158
C	233270	241259	274819	305120	37894	35918	13930	5.61E+03	1.22E+04	3774	6.35E+03	4063	6018	1.11E+06	27501	4.68E+04
G	211701	187534	185281	196614	66632	9.88E+05	26572	1074020	12936	9205	1066570	7418	1050360	3828	3949	2231
T	480761	461193	476317	387466	942707	37284	1.08E+06	19204	34885	1.87E+04	19450	11145	1.77E+04	13689	1.08E+06	1068370
1000 nM Cas9:CLTA3 v1.0 sgRNA																
position	−4	−3	−2	−1	1	2	3	4	5	6	7	8	9	10	11	12
A	219833	263464	207913	264018	97886	78562	20663	39724	1136320	1151200	42966	1156400	49443	18669	44652	44644
C	240570	261247	311444	333414	39996	40484	13961	5323	11099	5475	10323	6501	8456	1126310	36792	56203
G	221683	206195	199246	215583	76580	1032080	24785	1126840	12654	12465	1114450	12075	1113930	12078	19275	9014
T	506611	457791	470094	375682	974235	37571	1129290	16811	28626	19560	20956	13723	16864	31636	1087980	1078840
1000 nM Cas9:CLTA3 v2.1 sgRNA																
position	−4	−3	−2	−1	1	2	3	4	5	6	7	8	9	10	11	12
A	169775	206549	166197	201768	75243	67150	20449	36549	876154	898360	39901	911344	44415	13218	37301	33080
C	197800	209445	243688	264177	32775	34540	14250	7885	14793	4878	7791	4636	7510	890591	28425	46269
G	174766	158928	158824	168325	58121	801768	26558	866689	13343	12052	868394	8837	867980	7923	14022	6553
T	394073	361492	367705	302144	770275	32956	875157	25291	32124	21124	20328	11597	16509	24682	856666	850512

TABLE 2-continued

CLTA3 pre-selection library

position	−4	−3	−2	−1	1	2	3	4	5	6	7	8	9	10	11	12
A	173122	135327	138244	142599	50365	69486	37040	66315	575295	566722	70249	528947	72610	41265	61770	56547
C	143788	158534	162646	177240	25902	40142	28129	34668	38933	36129	61591	52201	46032	559715	32233	34830
G	137601	132826	130592	128304	42860	534378	42217	531723	29873	34068	479149	49753	501888	41949	43243	30118
T	238486	266310	261515	244854	573870	48991	585611	60291	48896	56078	82008	62096	72467	50068	555751	571502

100 nM Cas9:CLTA4 v2.1 sgRNA

position	−4	−3	−2	−1	1	2	3	4	5	6	7	8	9	10	11	12
A	55030	78101	78867	81833	8.09E+04	58148	525585	29962	544918	19446	54151	2.59E+04	550200	29521	34194	38891
C	168401	162082	139480	130495	22088	428628	4498	1.21E+04	5.14E+03	15601	7.10E+03	35217	2481	2.35E+04	16846	2.03E+04
G	89302	75785	82959	133275	415632	4.70E+04	14868	504358	6156	9951	493432	14899	4528	498832	27411	497382
T	248025	244790	259452	215155	42090	26956	1.58E+04	14300	4541	5.16E+05	6071	484788	3.55E+03	8877	4.82E+05	4222

1000 nM Cas9:CLTA4 v1.0 sgRNA

position	−4	−3	−2	−1	1	2	3	4	5	6	7	8	9	10	11	12
A	95188	141261	145156	141850	151224	116745	928773	50295	975924	29201	95476	30383	980248	50181	65094	77253
C	305024	297215	260676	243819	34420	745345	8606	17266	7541	29948	10779	47831	5069	32501	30389	29610
G	159888	139073	153474	225343	742232	85777	29776	907007	9285	13455	883325	19640	8303	902733	44730	879985
T	438973	421524	439767	388061	71197	51206	31918	24505	6323	926469	9493	901219	5453	13658	858860	12225

1000 nM Cas9:CLTA4 v2.1 sgRNA

position	−4	−3	−2	−1	1	2	3	4	5	6	7	8	9	10	11	12
A	47674	70467	71535	72698	72554	54587	471218	27627	493315	16818	47470	17728	498471	29769	40021	41618
C	154985	151636	133622	122579	18730	384037	4452	10916	4303	16232	5436	28594	1961	19017	19152	18001
G	80869	69972	76726	118084	379024	42360	14989	453870	5084	6863	448784	10260	3281	450120	23076	439828
T	222651	214104	224296	192818	35871	25195	15520	13766	3477	466266	4489	449597	2466	7273	423930	6732

CLTA4 pre−selection library

position	−4	−3	−2	−1	1	2	3	4	5	6	7	8	9	10	11	12
A	106798	131577	131941	132368	127160	103294	820923	103844	840417	99163	133349	123366	824537	126564	115133	122618
C	304597	297419	277233	283453	50833	722264	29748	65558	44890	59551	73916	77470	45318	84973	73106	90384
G	146240	137027	134399	183111	695802	68240	51484	708098	30709	62837	67352	89897	49093	672860	88125	663922
T	393868	385480	407930	352571	77708	57705	49348	74003	35487	729952	70486	660770	32555	67106	675139	74579

100 nM Cas9:CLTA1 v2.1 sgRNA

position	13	14	15	16	17	18	19	20	PAM1	PAM2	PAM3	+4	+5	+6	+7
A	8551	9668	4582	32237	1.19E+06	1.20E+06	2032	4237	261056	1386	574	235167	223887	222343	301956
C	1.14E+06	1.18E+06	4090	1.13E+06	4363	628	969	1.19E+06	210095	167	152	211027	273777	264354	309690

TABLE 2-continued

G	3294	3867	3597	7260	3400	2474	1.19E+06	1301	238989	1.20E+06	1.21E+06	205765	222282	240526	217260
T	57980	13065	1.19E+06	36826	8959	3966	9354	8065	496128	475	211	554309	486322	479045	377362
1000 nM Cas9:CLTA1 v1.0 sgRNA															
position	13	14	15	16	17	18	19	20	PAM1	PAM2	PAM3	+4	+5	+6	+7
A	7925	9269	4859	32891	910633	925527	3595	5976	183479	1390	413	182704	171051	174062	221899
C	880022	908816	4419	859691	5694	776	2120	920211	180463	120	88	180657	220438	211411	245967
G	2819	3185	2994	6763	3631	2894	916417	1415	193418	932808	934044	172551	172071	176484	161703
T	43918	13414	922412	35339	14726	5487	12552	7082	377324	366	139	398772	371124	372727	305115
1000 nM Cas9:CLTA1 v2.1 sgRNA															
position	13	14	15	16	17	18	19	20	PAM1	PAM2	PAM3	+4	+5	+6	+7
A	8961	19434	9549	35083	604115	607264	4665	16515	125225	10391	2519	125288	114575	120476	149847
C	594469	616112	11645	553993	13212	4438	5146	590160	116022	329	138	123802	154249	146572	166531
G	3378	3517	5896	22551	8658	12770	613580	3712	121392	628464	637588	118800	113560	118464	111278
T	33583	1328	613301	28764	14406	15919	17000	30004	277752	1207	146	272501	258007	254879	212735
CLTA1 pre-selection library															
position	13	14	15	16	17	18	19	20	PAM1	PAM2	PAM3	+4	+5	+6	+7
A	88029	109977	62686	119399	931093	908362	64248	111479	190574	97896	104002	183367	178912	198049	219754
C	841819	817157	51676	797914	60106	52998	42317	813253	239201	56843	59450	289074	295400	289007	284268
G	86080	96496	81367	104949	52143	77389	918970	96000	192652	879150	870948	196672	202194	196499	202544
T	113595	105893	933794	107261	86181	90774	103988	108791	507096	95634	95123	460410	453017	445968	422957
100 nM Cas9:CLTA2 v2.1 sgRNA															
position	13	14	15	16	17	18	19	20	PAM1	PAM2	PAM3	+4	+5	+6	+7
A	59160	36601	2974	12980	3.27E+03	1.09E+03	17686	689	193742	284	129	143150	165553	136708	146056
C	1.48E+04	9.12E+03	660929	6.50E+05	660305	666122	1314	6.65E+05	42664	48	43	162563	111729	143442	177253
G	581322	606454	1564	2134	1819	89	6.44E+05	505	137388	6.68E+05	6.68E+05	103305	146355	139972	124772
T	13024	16134	2.85E+03	3253	2918	1016	4886	2608	294518	146	42	259294	244675	248190	220231
1000 nM Cas9:CLTA2 v1.0 sgRNA															
position	13	14	15	16	17	18	19	20	PAM1	PAM2	PAM3	+4	+5	+6	+7
A	49577	39401	5425	30774	6408	5055	36081	2573	148145	782	243	132801	126862	118528	122897
C	13617	13316	563557	535780	560658	567693	4938	569653	46472	70	45	133402	123970	130555	148756
G	495156	496382	1789	3325	1846	166	519782	520	125177	575395	576103	118877	108849	104210	103370
T	18093	27344	5672	6564	7531	3529	15642	3697	256649	196	52	191363	216762	223150	201420

TABLE 2-continued

1000 nM Cas9:CLTA2 v2.1 sgRNA															
position	13	14	15	16	17	18	19	20	PAM1	PAM2	PAM3	+4	+5	+6	+7
A	32780	22855	9722	25181	12518	17950	28198	5471	100745	4933	834	89339	87351	82615	85108
C	9569	9710	374342	355544	373485	370343	11652	378841	40532	238	34	93621	87920	91380	105625
G	344511	350245	1559	5882	1339	391	331376	1034	74803	393760	398660	79776	75927	74068	70435
T	12700	16750	13937	12953	12218	10876	28334	14214	183480	629	32	136824	148362	151497	138392
CLTA2 pre-selection library															
position	13	14	15	16	17	18	19	20	PAM1	PAM2	PAM3	+4	+5	+6	+7
A	91515	84764	79586	86205	87337	85547	92983	100316	177716	84144	88017	177831	180209	176904	174190
C	49519	46571	641958	624548	637703	635473	51727	594349	136372	41282	41689	216880	206368	210039	235263
G	627263	642878	59549	55292	53056	57979	616575	66553	158929	656315	654970	162242	160704	157741	138890
T	79321	73405	66525	81573	69522	68619	86333	86400	374601	65877	62942	290665	300337	302934	299275
100 nM Cas9:CLT3A v2.1 sgRNA															
position	13	14	15	16	17	18	19	20	PAM1	PAM2	PAM3	+4	+5	+6	+7
A	6465	1130430	4097	5750	7.71E+04	1.14E+06	6151	2047	305062	1993	394	213566	240851	230230	252637
C	1.12E+06	1.96E+03	1129400	1.82E+03	3421	167	1451	6.66E+02	261609	103	82	319990	253055	261338	293644
G	2504	2471	1726	2881	1081680	876	1.13E+06	600	228865	1.14E+06	1.14E+06	142425	192720	220683	227840
T	9829	3709	3.34E+03	1128120	6398	1320	4480	1135260	343032	211	69	462587	451942	426317	364447
1000 nM Cas9:CLTA3 v1.0 sgRNA															
position	13	14	15	16	17	18	19	20	PAM1	PAM2	PAM3	+4	+5	+6	+7
A	44771	1152540	16264	30980	71714	1156700	47106	27658	276285	36304	12701	219034	239515	244440	255360
C	1096280	8437	1156840	8448	25120	4351	24685	9473	297135	1331	939	354289	298216	277740	292917
G	7707	9466	2708	17195	1053760	10278	1085310	10308	238545	1148550	1174510	171862	193096	217301	239319
T	39940	18250	12883	1132070	38103	17372	31596	1141260	376732	2514	550	443512	457870	449216	401101
1000 nM Cas9:CLTA3 v2.1 sgRNA															
position	13	14	15	16	17	18	19	20	PAM1	PAM2	PAM3	+4	+5	+6	+7
A	26409	893670	6315	20807	52541	903619	33690	20904	205940	26623	9880	172210	182986	187305	196429
C	870864	7991	910584	5931	19923	4977	18171	6508	229797	1163	693	283240	240802	224453	236469
G	3393	7912	1499	12906	836022	9011	859600	8302	190011	906628	925513	132620	153591	172169	187623
T	35748	26841	18016	896770	27928	18807	24953	900700	310666	2000	328	348344	359035	352487	315893
CLTA3 pre-selection library															
position	13	14	15	16	17	18	19	20	PAM1	PAM2	PAM3	+4	+5	+6	+7
A	75555	586476	61203	51740	70943	569277	70484	50807	130402	57527	61702	110207	118993	126967	127707
C	519328	30904	540977	24982	45344	35359	44014	35778	174938	42259	46083	201434	190347	184768	207347

TABLE 2-continued

G	38922	34282	34082	37275	515778	36956	516177	45203	137307	539445	527404	113323	119846	118423	127230
T	59192	41335	56735	579000	60932	51405	62322	561209	250350	53766	57808	268033	263811	262839	230713
100 nM Cas9:CLTA4 v2.1 sgRNA															
position	13	14	15	16	17	18	19	20	PAM1	PAM2	PAM3	+4	+5	+6	+7
A	26542	23991	15243	25122	5.36E+03	5.51E+05	1994	540029	47731	4642	1401	77633	56902	63224	54815
C	3.69E+04	8.47E+03	5182	5.22E+05	547711	5715	546119	3.02E+03	152056	655	473	141123	164035	146401	190955
G	6729	3344	3716	3926	3162	554	1.45E+03	4637	72296	5.55E+05	5.58E+05	84257	77627	75123	91454
T	490573	524958	5.37E+05	9437	4528	3692	11194	13069	288675	911	495	257745	262194	276010	223534
1000 nM Cas9:CLTA4 v1.0 sgRNA															
position	13	14	15	16	17	18	19	20	PAM1	PAM2	PAM3	+4	+5	+6	+7
A	42674	41050	32933	55244	39984	942989	19900	887311	80159	28536	12390	142460	96664	110844	99920
C	61641	25910	21400	887446	900777	34590	940504	23749	257985	2556	4791	252462	297152	258929	338099
G	16677	7879	8429	12432	17373	4103	7346	20095	139488	964013	976818	154302	139784	136512	165750
T	878081	924234	936311	43951	40939	17391	31323	67918	521441	3968	5074	449849	465473	492788	395304
1000 nM Cas9:CLTA4 v2.1 sgRNA															
position	13	14	15	16	17	18	19	20	PAM1	PAM2	PAM3	+4	+5	+6	+7
A	24741	23050	16409	27974	2697	478335	12667	451298	36128	22041	16967	68943	49017	56451	51102
C	35213	12845	13497	445302	480543	15631	469503	11832	122541	3529	8965	126313	153105	134293	171499
G	7741	5091	5456	7558	7112	3083	5302	10184	87517	474540	471647	85849	72063	71600	85239
T	438484	465193	470817	25345	15827	9130	18707	32865	259993	6069	8600	225074	231994	243835	198339
CLTA4 pre-selection library															
position	13	14	15	16	17	18	19	20	PAM1	PAM2	PAM3	+4	+5	+6	+7
A	108492	107761	96384	99908	76163	806675	75877	793806	87755	82110	83605	111015	103082	109315	101198
C	78280	76978	66776	738550	776738	55522	754283	42188	278802	57603	55530	266156	281433	258029	295144
G	67768	53472	58440	47550	41427	42574	54424	59162	151536	740525	732891	163824	158224	146268	151560
T	696963	713292	729903	65495	57175	46732	66919	56347	433410	71265	79477	410508	408764	437891	403601

TABLE 3

			no sgRNA		v1.0 sgRNA		v2.1 sgRNA	
	m	sequence	modified sequences	total sequences	modified sequences	total sequences	modified sequences	total sequences
CLTA1-0-1	0	AGTCCTCATCTCCCTCAAGCAGG (SEQ ID NO: 91)	2	58889	18	42683	178	52845
CLTA1-1-1	1	AGTCCTCAaCTCCCTCAAGCAGG (SEQ ID NO: 92)	1	39804	9	29000	37	40588
CLTA1-2-1	2	AGcCCTCATtTCCCTCAAGCAGG (SEQ ID NO: 93)	0	16276	0	15032	0	18277
CLTA1-2-2	2	AcTCCTCATCcCCCTCAAGCCGG (SEQ ID NO: 94)	3	21267	1	20042	33	22579
CLTA1-2-3	2	AGTCaTCATCTCCCTCAAGCAGa (SEQ ID NO: 95)	0	0	0	0	0	0
CLTA1-3-1	3	cGTCCTCcTCTCCCcCAAGCAGG (SEQ ID NO: 96)	2	53901	0	42194	0	52205
CLTA1-3-2	3	tGTCCTCtTCTCCCTCAAGCAGa (SEQ ID NO: 97)	0	14890	0	14231	0	15937
CLTA1-4-1	4	AagCtTCATCTCtCTCAAGCTGG (SEQ ID NO: 98)	0	49579	2	31413	0	41234
CLTA1-4-2	4	AGTaCTCtTtTCCCTCAgGCTGG (SEQ ID NO: 99)	2	30013	1	23470	4	26999
CLTA1-4-3	4	AGTCtTaAatTCCCTCAAGCAGG (SEQ ID NO: 100)	2	63792	0	52321	1	73007
CLTA1-4-4	4	AGTgCTCATCTaCCagAAGCTGG (SEQ ID NO: 101)	1	12585	0	11339	0	12066
CLTA1-4-5	4	ccTCCTCATCTCCCTgcAGCAGG (SEQ ID NO: 102)	4	30568	1	23810	0	27870
CLTA1-4-6	4	ctaCaTCATCTCCCTCAAGCTGG (SEQ ID NO: 103)	0	13200	1	12886	2	12843
CLTA1-4-7	4	gGTCCTCATCTCCCTaAAaCAGa (SEQ ID NO: 104)	1	8697	3	8188	0	8783
CLTA1-4-8	4	tGTCCTCATCggCCTCAgGCAGG (SEQ ID NO: 105)	0	13169	0	8805	2	12830
CLTA1-5-1	5	AGaCacCATCTCCCTtgAGCTGG (SEQ ID NO: 106)	0	46109	1	32515	2	35567
CLTA1-5-2	5	AGgCaTCATCTaCaTCAAGtTGG (SEQ ID NO: 107)	0	41280	0	28896	0	35152
CLTA1-5-3	5	AGTaaTCActTCCaTCAAGCCGG (SEQ ID NO: 108)	0	0	0	0	0	0
CLTA1-5-4	5	tccCCTCAcCTCCCTaAAGCAGG (SEQ ID NO: 109)	2	24169	5	17512	1	23483
CLTA1-5-5	5	tGTCtTtATtTCCCTCtAGCTGG (SEQ ID NO: 110)	0	11527	0	10481	1	11027
CLTA1-6-1	6	AGTCCTCATCTCCCTCAAGCAGG (SEQ ID NO: 111)	0	6537	0	5654	0	6741

TABLE 4

		no sgRNA		v1.0 sgRNA		v2.1 sgRNA	
	m sequence	modified sequences	total sequences	modified sequences	total sequences	modified sequences	total sequences
CLTA4-0-1	0 GCAGATGTAGTGTTTCCACAGGG (SEQ ID NO: 112)	6	29191	2005	18640	14970	19661
CLTA4-3-1	3 aCAtATGTAGTaTTTCCACAGGG (SEQ ID NO: 113)	2	34165	11	20018	3874	16082
CLTA4-3-2	3 GCAtATGTAGTGTTTCCAaATGt (SEQ ID NO: 114)	3	17923	0	11688	2	13880
CLTA4-3-3	3 cCAGATGTAGTaTTcCCACAGGG (SEQ ID NO: 115)	0	16559	0	12007	52	11082
CLTA4-3-4	3 GCAGtTtTAGTGTTTtCACAGGG (SEQ ID NO: 116)	0	21722	0	12831	0	15726
CLTA4-3-5	3 GCAGAgtTAGTGTTTCCACACaG (SEQ ID NO: 117)	1	21222	2	13555	3	16425
CLTA4-3-6	3 GCAGATGgAGgGTTTtCACAGGG (SEQ ID NO: 118)	3	20342	3	12804	3	14068
CLTA4-3-7	3 GgAaATtTAGTGTTTCCACAGGG (SEQ ID NO: 119)	2	38894	3	24017	1	29347
CLTA4-4-1	4 aaAGAaGTAGTaTTTCCACATGG (SEQ ID NO: 120)	0	0	0	0	0	0
CLTA4-4-2	4 aaAGATGTAGTcaTTCCACAAGG (SEQ ID NO: 121)	1	27326	0	17365	1	18941
CLTA4-4-3	4 aaAtATGTAGTcTTTCCACAGGG (SEQ ID NO: 122)	2	46232	3	32264	0	32638
CLTA4-4-4	4 atAGATGTAGTGTTTCCAaAGGa (SEQ ID NO: 123)	9	27821	1	16223	8	15388
CLTA4-4-5	4 cCAGAgGTAGTGcTcCCACAGGG (SEQ ID NO: 124)	1	20979	1	15674	1	15086
CLTA4-4-6	4 cCAGATGTgagGTTTCCACAAGG (SEQ ID NO: 125)	4	22021	0	15691	1	14253
CLTA4-4-7	4 ctAcATGTAGTGTTTCCAtATGG (SEQ ID NO: 126)	2	35942	0	23076	1	11867
CLTA4-4-8	4 ctAGATGaAGTGcTTCCACATGG (SEQ ID NO: 127)	1	10692	1	7609	59	8077
CLTA4-4-9	4 GaAaATGgAGTGTTTaCACATGG (SEQ ID NO: 128)	0	34616	0	22302	1	24671
CLTA4-4-10	4 GCAaATGaAGTGTcaCCACAAGG (SEQ ID NO: 129)	1	25210	0	16187	0	16974
CLTA4-4-11	4 GCAaATGTAtTaTTTCCACtAGG (SEQ ID NO: 130)	0	34144	1	24770	0	22547
CLTA4-4-12	4 GCAGATGTAGctTTTgtACATGG (SEQ ID NO: 131)	0	14254	0	9616	0	9994
CLTA4-4-13	4 GCAGcTtaAGTGTTTtCACATGG (SEQ ID NO: 132)	8	39466	1	7609	5	16525
CLTA4-4-14	4 ttAcATGTAGTGTTTaCACACGG (SEQ ID NO: 133)	0	0	0	22302	0	0
CLTA4-5-1	5 GaAGAgGaAGTGTTTgCcCAGGG (SEQ ID NO: 134)	1	27616	1	16319	1	16140
CLTA4-5-2	5 GaAGATGTgGaGTTgaCACATGG (SEQ ID NO: 135)	1	22533	0	14292	0	15013
CLTA4-5-3	5 GCAGAaGTAcTGTTgttACAAGG (SEQ ID NO: 136)	1	44243	1	29391	1	29734

TABLE 4-continued

	m	sequence	no sgRNA modified sequences	no sgRNA total sequences	v1.0 sgRNA modified sequences	v1.0 sgRNA total sequences	v2.1 sgRNA modified sequences	v2.1 sgRNA total sequences
CLTA4-5-4	5	GCAGATGTgGaaTTaCaACAGGG (SEQ ID NO: 137)	0	27321	0	13640	0	14680
CLTA4-5-5	5	GCAGtcaTAGTGTaTaCACATGG (SEQ ID NO: 138)	1	26538	0	18449	1	20559
CLTA4-5-6	5	taAGATGTAGTaTTTCCAaAAGt (SEQ ID NO: 139)	1	15145	1	8905	0	7911
CLTA4-6-1	6	GCAGcTGgcaTtTcTCCACACGG (SEQ ID NO: 140)	0	2	0	0	0	0
CLTA4-6-2	6	GgAGATcTgaTGgTTCtACAAGG (SEQ ID NO: 141)	2	27797	0	19450	2	21709
CLTA4-6-3	6	taAaATGcAGTGTaTCCAtATGG (SEQ ID NO: 142)	4	27551	0	18424	0	18783
CLTA4-7-1	7	GCcagaaTAGTtTTTCaACAAGG (SEQ ID NO: 143)	0	20942	0	13137	1	13792
CLTA4-7-2	8	ttgtATtTAGaGaTTgCACAAGG (SEQ ID NO: 144)	0	28470	0	18104	0	20416

TABLE 5

Off-target site	Human genome coordinates
CLTA1-0-1	9(+): 36,211,732-36,211,754
	12(+): 7,759,893-7,759,915
CLTA1-1-1	8(−): 15,546,437-15,546,459
CLTA1-2-1	3(−): 54,223,111-54,223,133
CLTA1-2-2	15(+): 89,388,670-89,388,692
CLTA1-2-3	5(+): 88716920-88,716,942
CLTA1-3-1	21(+): 27,972,462-27,972,484
CLTA1-3-2	4(−): 17,179,924-17,179,946
CLTA1-4-1	1(+): 147,288,742-147,288,764
CLTA1-4-2	10(+): 97,544,444-97,544,466
CLTA1-4-3	2(−): 161,873,870-161,873,892
CLTA1-4-4	1(+): 196,172,702-196,172,724
CLTA1-4-5	13(+): 56,574,636-56,574,658
CLTA1-4-6	2(+): 241,357,827-241,357,849
CLTA1-4-7	3(+): 121,248,627-121,248,649
CLTA1-4-8	12(+): 132,937,319-132,937,341
CLTA1-5-1	9(−): 80,930,919-80,930,941
CLTA1-5-2	2(+): 140,901,875-14,0901,897
CLTA1-5-3	3(+): 45,016,841-45,016,863
CLTA1-5-4	X(+): 40,775,684-40,775,706
CLTA1-5-5	2(−): 185,151,622-185,151,644
CLTA1-6-1	X(+): 150,655,097-150,655,119
CLTA4-0-1	9(−): 36,211,779-36,211,801
CLTA4-3-1	12(−): 50,679,419-50,679,441
CLTA4-3-2	X(−): 143,939,483-143,939,505
CLTA4-3-3	11(−): 47,492,611-47,492,633
CLTA4-3-4	3(−): 162,523,715-162,523,737
CLTA4-3-5	11(+): 30,592,975-30,592,997

TABLE 5-continued

Off-target site	Human genome coordinates
CLTA4-3-6	4(−): 155,252,699-155,252,721
CLTA4-3-7	18(+): 39,209,441-39,209,463
CLTA4-4-1	17(−): 36,785,650-36,785,672
CLTA4-4-2	1(−): 241,537,119-241,537,141
CLTA4-4-3	8(−): 120,432,103-120,432,125
CLTA4-4-4	6(−): 106,204,600-106,204,622
CLTA4-4-5	8(+): 102,527,804-102,527,826
CLTA4-4-6	8(−): 94,685,538-94,685,560
CLTA4-4-7	2(+): 35,820,054-35,820,076
CLTA4-4-8	3(−): 36,590,352-36,590,374
CLTA4-4-9	12(+): 100,915,498-100,915,520
CLTA4-4-10	21(+): 33,557,705-33,557,727
CLTA4-4-11	8(+): 10,764,183-10,764,205
CLTA4-4-12	19(+): 37,811,645-37,811,667
CLTA4-4-13	13(−): 26,832,673-26,832,695
CLTA4-4-14	6(+): 19,349,572-19,349,594
CLTA4-5-1	11(−): 502,300-502,322
CLTA4-5-2	8(−): 28,389,683-28,389,705
CLTA4-5-3	2(−): 118,557,405-118,557,427
CLTA4-5-4	2(−): 103,248,360-103,248,382
CLTA4-5-5	21(−): 42,929,085-42,929,107
CLTA4-5-6	13(−): 83,097,278-83,097,300
CLTA4-6-1	2(+): 43,078,423-43,078,445
CLTA4-6-2	7(−): 11,909,384-11,909,406
CLTA4-6-3	5(−): 69,775,482-69,775,504
CLTA4-7-1	16(+): 30,454,945-30,454,967
CLTA4-7-2	9(−): 77,211,328-77,211,350

TABLE 6

	number of mutations	sequence	gene	in vitro enrichment v1.0	in vitro enrichment v2.1	modification frequency in HEK293T cells no sgRNA	modification frequency in HEK293T cells v1.0	modification frequency in HEK293T cells v2.1
CLTA1-0-1	0	AGTCCTCATCTCCCTCAAGCAGG (SEQ ID NO: 145)	CLTA	41.4	23.3	0.003%	0.042%	0.337%
CLTA1-1-1	1	AGTCCTCAaCTCCCTCAAGCAGG (SEQ ID NO: 146)	TUSC3	25.9	14	0.003%	0.031%	0.091%

TABLE 6-continued

	number of mutations	sequence	gene	in vitro enrichment v1.0	in vitro enrichment v2.1	modification frequency in HEK293T cells no sgRNA	modification frequency in HEK293T cells v1.0	modification frequency in HEK293T cells v2.1
CLTA1-2-1	2	AGcCCTCATtTCCCTCAAGCAGG (SEQ ID NO: 147)	CACNA2D3	15.4	26.2	0%	0%	0%
CLTA1-2-2	2	AcTCCTCATCcCCCTCAAGCCGG (SEQ ID NO: 148)	ACAN	29.2	18.8	0.014%	0.005%	0.146%
CLTA1-2-3	2	AGTCaTCATCTCCCTCAAGCAGa (SEQ ID NO: 149)		0.06	1.27	n.t.	n.t.	n.t.
CLTA1-3-1	3	cGTCCTCcTCTCCCcCAAGCAGG (SEQ ID NO: 150)		0	2.07	0.004%	0%	0%
CLTA1-3-2	3	tGTCCTCtTCTCCCTCAAGCAGa (SEQ ID NO: 151)	BC029598	0	1.47	0%	0%	0%
CLTA1-4-1	4	AagCtTCATCTCtCTCAAGCTGG (SEQ ID NO: 152)				0%	0.006%	0%
CLTA1-4-2	4	AGTaCTCtTtTCCCTCAgGCTGG (SEQ ID NO: 153)	ENTPD1			0.007%	0.004%	0.015%
CLTA1-4-3	4	AGTCtTaAatTCCCTCAAGCAGG (SEQ ID NO: 154)				0.003%	0%	0.001%
CLTA1-4-4	4	AGTgCTCATCTaCCagAAGCTGG (SEQ ID NO: 155)				0.008%	0%	0%
CLTA1-4-5	4	ccTCCTCATCTCCCTgcAGCAGG (SEQ ID NO: 156)				0.013%	0.004%	0%
CLTA1-4-6	4	ctaCaTCATCTCCCTCAAGCTGG (SEQ ID NO: 157)				0%	0.008%	0.016%
CLTA1-4-7	4	gGTCCTCATCTCCCTaAAaCAGa (SEQ ID NO: 158)	POLQ (coding)			0.011%	0.037%	0%
CLTA1-4-8	4	tGTCCTCATCggCCTCAgGCAGG (SEQ ID NO: 159)				0%	0%	0.016%
CLTA1-5-1	5	AGaCacCATCTCCCTtgAGCTGG (SEQ ID NO: 160)	PSAT1			0%	0.003%	0.006%
CLTA1-5-2	5	AGgCaTCATCTaCaTCAAGtTGG (SEQ ID NO: 161)				0%	0%	0%
CLTA1-5-3	5	AGTaaTCActTCCaTCAAGCCGG (SEQ ID NO: 162)	ZDHHC3, EXOSC7			n.t.	n.t.	n.t.
CLTA1-5-4	5	tccCCTCAcCTCCCTaAAGCAGG (SEQ ID NO: 163)				0.008%	0.029%	0.004%
CLTA1-5-5	5	tGTCtTtATtTCCCTCtAGCTGG (SEQ ID NO: 164)				0%	0%	0.009%
CLTA1-6-1	6	AGTCCTCATCTCCCTCAAGCAGG (SEQ ID NO: 165)				0%	0%	0%

TABLE 7

sequence	# of sequences no sgRNA	# of sequences v1.0 sgRNA	# of sequences v2.1 sgRNA
CLTA1-0-1			
ref AGTCCTCATCTCCCTCAAGCAGG (SEQ ID NO: 166)	58,887	42,665	52,667
AGTCCTCATCTCCCTCA**A**AGCAGG (SEQ ID NO: 167)	0	0	66
AGTCCTCATCTCCCTC-AGCAGG (SEQ ID NO: 168)	0	2	28

TABLE 7-continued

		# of sequences		
	sequence	no sgRNA	v1.0 sgRNA	v2.1 sgRNA
	AGTCCTCAT----------	0	0	13
	AGTCCTCATCTCCCTCA**T**AGCAGG (SEQ ID NO: 169)	0	0	11
	AGTCCTCAT--------AGCAGG (SEQ ID NO: 170)	0	0	9
	AGTCCTCATCT------AGCAGG (SEQ ID NO: 171)	0	0	8
	AGTCCTCA---------AGCAGG (SEQ ID NO: 172)	0	0	6
	AGTCCTCATCTCCCTCA**AAGGCAGTGTTTGTT ACTTGAGTTTGTC**AGCAGG (SEQ ID NO: 173)	0	0	4
	AGTCCTCATCTCCCTCA**TT**AGCAGG (SEQ ID NO: 174)	0	0	4
	AGTCCTCATCTCCCTCA**GGGCTTGTTTACAGC TCACCTTTGAATTTGCACAAGCGTGCA**AGCAG G (SEQ ID NO: 175)	0	0	3
	AGTCCTCATCTCCCT-AGCAGG (SEQ ID NO: 176)	0	11	0
	AGTCCTCATCCCTC-AAGCAGG (SEQ ID NO: 177)	0	3	0
	AGTCCTCATCTCCCT-AAGCAGG (SEQ ID NO: 178)	1	2	0
	other	1	0	26
	modified total	2	18 (0.042%)	178 (0.34%)
	CLTA1-1-1			
ref	AGTCCTCATCTCCCTCAAGCAGG (SEQ ID NO: 179)	39,803	28,991	40,551
	AGTCCTCAaCTCCCTCA**A**AGCAGG (SEQ ID NO: 180)	0	4	13
	AGTCCTCAaCTCCCTCA------ (SEQ ID NO: 181)	0	0	12
	AGTCCTCAaCTCCCTC-AGCAGG (SEQ ID NO: 182)	0	2	4
	AGTCCTCAaCTCCCTCA**AGAAAGGTGTTGAAAA TCAGAAAGAGAGAAACA**AGCAGG (SEQ ID NO: 183)	0	0	3
	AGTCCTCAaCTCCCTCA**ATCTACGGTCCATTCC CGTTTCCACTCACCTTGCGCCGC**AGCAGG (SEQ ID NO: 184)	0	0	2
	AGTCCTCAaCTCCCT-AAGCAGG (SEQ ID NO: 185)	0	3	1
	AGTCCTCAaCTCCCTCA**ACCAACTTTAACATCC TGCTGGTTCTGTCATTAATAAGTTGAA**AGCAGG (SEQ ID NO: 186)	0	0	1
	AGTCCTCAaCTCCCTCA**CAGCAAATAAAAAAGT TGTTTATGCATATTCAGATAAGCAA**AGCAGG (SEQ ID NO: 187)	0	0	1
	AGTCCTCAaCTCCC-AAGCAGG (SEQ ID NO: 188)	1	0	0
	modified total	1	9 (0.031%)	37 (0.091%)
	CLTA1-2-2			
ref	AcTCCTCATCcCCCTCAAGCCGG (SEQ ID NO: 189)	21,264	20,041	22,546
	AcTCCTCATCcCCCTCA**A**AGCCGG (SEQ ID NO: 190)	0	0	8
	AcTCCTCATCcCCCTCA**G**AGCCGG (SEQ ID NO: 191)	0	0	7
	AcTCCTC-----------AGCCGG (SEQ ID NO: 192)	0	0	5
	AcTCCTCATCcCCCTCA**AA**AGCCGG (SEQ ID NO: 193)	0	0	2
	AcTCCTCATCcCCCTCA**GC**AGCCGG (SEQ ID NO: 194)	0	0	2
	AcTCCTCATCcCCCTCA**T**AGCCGG (SEQ ID NO: 195)	0	0	2
	AcTCCTCATCcCCCTCA**TCC**CCGG (SEQ ID NO: 196)	0	0	2

TABLE 7-continued

sequence	# of sequences: no sgRNA	v1.0 sgRNA	v2.1 sgRNA
AcTCCTCATCcC-----AGCCGG (SEQ ID NO: 197)	0	0	2
AcTCCTCATCcCCCTA-AGCCGG (SEQ ID NO: 198)	3	1	1
AcTCCTCATCcCCCTCA**AT**AGCCGG (SEQ ID NO: 199)	0	0	1
AcTCCTCACCcCCCTCA**GC**AGCCGG (SEQ ID NO: 200)	0	0	1
modified total	3	1	33 (0.15%)

TABLE 8

	sequence	# of sequences: control	v1.0 sgRNA	v2.1 sgRNA
	CLTA4-0-1			
ref	GCAGATGTAGTGTTTCCACAGGG (SEQ ID NO: 201)	29,185	16,635	17,555
	GCAGATGTAGTGTTTC-ACAGGG (SEQ ID NO: 202)	1	891	5,937
	GCAGATGTAGTGTTTCC**C**ACAGGG (SEQ ID NO: 203)	0	809	5,044
	GCAGATGTAGTG----CACAGGG (SEQ ID NO: 204)	0	14	400
	GCAGATGTAGTGTTTCC-CAGGG (SEQ ID NO: 205)	0	19	269
	GCAGATGTAC-------ACAGGG (SEQ ID NO: 206)	0	17	262
	GCAGATGTAGTGTCA---CAGGG (SEQ ID NO: 207)	2	6	254
	GCAGATGTAGTGTTCA-CAGGG (SEQ ID NO: 208)	0	21	229
	GCAGATGTAGTGTTTC-CAGGG (SEQ ID NO: 209)	1	14	188
	GCAGATGTAGT-----CACAGGG (SEQ ID NO: 210)	0	0	152
	GCAGATGT----------AGGG (SEQ ID NO: 211)	0	6	129
	other	2	208	2,106
	modified total	6	2,005 (11%)	14,970 (76%)
	CLTA4-3-1			
ref	aCAtATGTAGTaTTTCCACAGGG (SEQ ID NO: 212)	34,163	20,007	12,208
	aCAtATGTAGTaTTTCC**C**ACAGGG (SEQ ID NO: 213)	0	8	1779
	aCAtATGTAGTaTTTCA-CAGGG (SEQ ID NO: 214)	1	0	293
	aCAtATGTAGTaTTTC-CAGGG (SEQ ID NO: 215)	1	0	227
	aCAtAT----------CACAGGG (SEQ ID NO: 216)	0	0	117
	a----------CAGGG	0	0	96
	aCAt----------CACAGGG (SEQ ID NO: 217)	0	0	78
	aCAtATGTAGT-----CACAGGG (SEQ ID NO: 218)	0	0	77
	aCAtATGTAGTaTTTCC------ (SEQ ID NO: 219)	0	0	76

TABLE 8-continued

	# of sequences		
sequence	control	v1.0 sgRNA	v2.1 sgRNA
aCAtATGT----------AGGG (SEQ ID NO: 220)	0	0	68
aCAtATGTAG------CACAGGG (SEQ ID NO: 221)	0	0	64
other	0	3	999
modified total	2	11 (0.055%)	3874 (24%)
CLTA4-3-3			
ref cCAGATGTAGTaTTcCCACAGGG (SEQ ID NO: 222)	16,559	12,007	11,030
cCAGATGTAGTaTTcCC**C**ACAGGG (SEQ ID NO: 223)	0	0	35
cCAGATGTAGTaT----ACAGGG (SEQ ID NO: 224)	0	0	5
cCAGATGTAGTaT---CACAGGG (SEQ ID NO: 225)	0	0	3
cCAGATGTAGTaTTcCC**AAC**ACAGGG (SEQ ID NO: 226)	0	0	2
cCAGATGTAGTaTT-CACAGGG (SEQ ID NO: 227)	0	0	2
cCAGATGTAGTaTTcC-CAGGG (SEQ ID NO: 228)	0	0	2
cCAGATGTA----------	0	0	2
cCAGATGTAGTaTTcC-ACAGGG (SEQ ID NO: 229)	0	0	1
modified total	0	0	52 (0.47%)
CLTA4-4-8			
ref ctAGATGaAGTGcTTCCACATGG (SEQ ID NO: 230)	10,691	7,608	8,018
ctAGATGaAGTGcTTCC**C**ACATGG (SEQ ID NO: 231)	0	0	49
ctAGATGaAGTGcTTC-ACATGG (SEQ ID NO: 232)	0	0	6
ctAGATGaAGTG---------- (SEQ ID NO: 233)	0	0	2
ctAGATGaAGTGcTTCCAC**AC**ATGG (SEQ ID NO: 234)	0	0	1
ctAGATGaAGTGcTTC-CATGG (SEQ ID NO: 235)	1	0	0
ctAGATGaAGTGcTTCC-CATGG (SEQ ID NO: 236)	0	1	0
modified total	1	1	59 (0.73%)

TABLE 9

oligonucleotide name	oligonucleotide sequence (5'->3')
CLTA1 v2.1 template fwd	TAA TAC GAC TCA CTA TAG GAG TCC TCA TCT CCC TCA AGC GTT TTA GAG CTA TGC TG (SEQ ID NO: 237)
CLTA2 v2.1 template fwd	TAA TAC GAC TCA CTA TAG GCT CCC TCA AGC AGG CCC CGC GTT TTA GAG CTA TGC TG (SEQ ID NO: 238)
CLTA3 v2.1 template fwd	TAA TAC GAC TCA CTA TAG GTG TGA AGA GCT TCA CTG AGT GTT TTA GAG CTA TGC TG (SEQ ID NO: 239)
CLTA4 v2.1 template fwd	TAA TAC GAC TCA CTA TAG GGC AGA TGT AGT GTT TCC ACA GTT TTA GAG CTA TGC TG (SEQ ID NO: 240)
v2.1 template rev	GAT AAC GGA CTA GCC TTA TTT TAA CTT GCT ATG CTT TTC AGC ATA GCT CTA AAA C (SEQ ID NO: 241)
CLTA1 v1.0 template	CGG ACT AGC CTT ATT TTA ACT TGC TAT TTC TAG CTC TAA AAC GCT TGA GGG AGA TGA GGA CTC CTA TAG TGA GTC GTA TTA (SEQ ID NO: 242)
CLTA2 v1.0 template	CGG ACT AGC CTT ATT TTA ACT TGC TAT TTC TAG CTC TAA AAC GCG GGG CCT GCT TGA GGG AGC CTA TAG TGA GTC GTA TTA (SEQ ID NO: 243)
CLTA3 v1.0 template	CGG ACT AGC CTT ATT TTA ACT TGC TAT TTC TAG CTC TAA AAC ACT CAG TGA AGC TCT TCA CAC CTA TAG TGA GTC GTA TTA (SEQ ID NO: 244)
CLTA4 v1.0 template	CGG ACT AGC CTT ATT TTA ACT TGC TAT TTC TAG CTC TAA AAC TGT GGA AAC ACT ACA TCT GCC CTA TAG TGA GTC GTA TTA (SEQ ID NO: 245)
T7 promoter oligo	TAA TAC GAC TCA CTA TAG G (SEQ ID NO: 246)
CLTA1 lib	/5Phos/AAC ACA NNN NC*C* NG*C* T*T*G* A*G*G* G*A*G* A*T*G* A*G*G* A*C*T* NNN NAC CTG CCG AGA ACA CA (SEQ ID NO: 247)
CLTA2 lib	/5Phos/TCT TCT NNN NC*C* NG*C* G*G*G* G*C*C* T*G*C* T*T*G* A*G*G* G*A*G* NNN NAC CTG CCG AGT CTT CT (SEQ ID NO: 248)
CLTA3 lib	/5Phos/AGA GAA NNN NC*C* NA*C* T*C*A* G*T*G* A*A*G* C*T*C* T*T*C* A*C*A* NNN NAC CTG CCG AGA GAG AA (SEQ ID NO: 249)
CLTA4 lib	/5Phos/TTG TGT NNN NC*C* NT*G* T*G*G* A*A*A* C*A*C* T*A*C* A*T*C* T*G*C* NNN NAC CTG CCG AGT TGT GT (SEQ ID NO: 250)
CLTA1 site fwd	CTA GCA GTC CTC ATC TCC CTC AAG CAG GC (SEQ ID NO: 251)
CLTA1 site rev	AGC TGC CTG CTT GAG GGA GAT GAG GAC TG (SEQ ID NO: 252)
CLTA2 site fwd	CTA GTC TCC CTC AAG CAG GCC CCG CTG GT (SEQ ID NO: 253)
CLTA2 site rev	AGC TAC CAG CGG GGC CTG CTT GAG GGA GA (SEQ ID NO: 254)
CLTA3 site fwd	CTA GCT GTG AAG AGC TTC ACT GAG TAG GA (SEQ ID NO: 255)
CLTA3 site rev	AGC TTC CTA CTC AGT GAA GCT CTT CAC AG (SEQ ID NO: 256)
CLTA4 site fwd	CTA GTG CAG ATG TAG TGT TTC CAC AGG GT (SEQ ID NO: 257)
CLTA4 site rev	AGC TAC CCT GTG GAA ACA CTA CAT CTG CA (SEQ ID NO: 258)
test fwd	GCG ACA CGG AAA TGT TGA ATA CTC AT (SEQ ID NO: 259)

TABLE 9-continued

oligonucleotide name	oligonucleotide sequence (5'->3')
test rev	GGA GTC AGG CAA CTA TGG ATG AAC G (SEQ ID NO: 260)
off-target CLTA4-0 fwd	ACT GTG AAG AGC TTC ACT GAG TAG GAT TAA GAT ATT GCA GAT GTA GTG TTT CCA CAG GGT (SEQ ID NO: 261)
off-target CLTA4-1 fwd	ACT GTG AAG AGC TTC ACT GAG TAG GAT TAA GAT ATT GAA GAT GTA GTG TTT CCA CAG GGT (SEQ ID NO: 262)
off-target CLTA4-2a fwd	ACT GTG AAG AGC TTC ACT GAG TAG GAT TAA GAT ATT GAA GAT GTA GTG TTT CCA CTG GGT (SEQ ID NO: 263)
off-target CLTA4-2b fwd	ACT GTG AAG AGC TTC ACT GAG TAG GAT TAA GAT ATT GCA GAT GGA GGG TTT CCA CAG GGT (SEQ ID NO: 264)
off-target CLTA4-2c fwd	ACT GTG AAG AGC TTC ACT GAG TAG GAT TAA GAT ATT GCA GAT GTA GTG TTA CCA GAG GGT (SEQ ID NO: 265)
off-target CLTA4-3 fwd	ACT GTG AAG AGC TTC ACT GAG TAG GAT TAA GAT ATT GGG GAT GTA GTG TTT CCA CTG GGT (SEQ ID NO: 266)
off-target CLTA4-0 rev	TCC CTC AAG CAG GCC CCG CTG GTG CAC TGA AGA GCC ACC CTG TGG AAA CAC TAC ATC TGC (SEQ ID NO: 267)
off-target CLTA4-1 rev	TCC CTC AAG CAG GCC CCG CTG GTG CAC TGA AGA GCC ACC CTG TGG AAA CAC TAC ATC TTC (SEQ ID NO: 268)
off-target CLTA4-2a rev	TCC CTC AAG CAG GCC CCG CTG GTG CAC TGA AGA GCC ACC CAG TGG AAA CAC TAC ATC TTC (SEQ ID NO: 269)
off-target CLTA4-2b rev	TCC CTC AAG CAG GCC CCG CTG GTG CAC TGA AGA GCC ACC CTG TGG AAA CCC TCC ATC TGC (SEQ ID NO: 270)
off-target CLTA4-2c rev	TCC CTC AAG CAG GCC CCG CTG GTG CAC TGA AGA GCC ACC CTC TGG TAA CAC TAC ATC TGC (SEQ ID NO: 271)
off-target CLTA4-3 rev	TCC CTC AAG CAG GCC CCG CTG GTG CAC TGA AGA GCC ACC CAG TGG AAA CAC TAC ATC CCC (SEQ ID NO: 272)
adapter1(AACA)	AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TAA CA (SEQ ID NO: 273)
adapter2(AACA)	TGT TAG ATC GGA AGA GCG TCG TGT AGG GAA AGA GTG TAG ATC TCG GTG G (SEQ ID NO: 274)
adapter1(TTCA)	AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TTT CA (SEQ ID NO: 275)
adapter2(TTCA)	TGA AAG ATC GGA AGA GCG TCG TGT AGG GAA AGA GTG TAG ATC TCG GTG G (SEQ ID NO: 276)
adapter1	AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC T (SEQ ID NO: 277)
adapter2	AGA TCG GAA GAG CGT CGT GTA GGG AAA GAG TGT AGA TCT CGG TGG (SEQ ID NO: 278)
lib adapter1	GAC GGC ATA CGA GAT (SEQ ID NO: 279)
CLTA1 lib adapter2	AAC AAT CTC GTA TGC CGT CTT CTG CTT G (SEQ ID NO: 280)
CLTA2 lib adapter2	TCT TAT CTC GTA TGC CGT CTT CTG CTT G (SEQ ID NO: 281)
CLTA3 lib adapter2	AGA GAT CTC GTA TGC CGT CTT CTG CTT G (SEQ ID NO: 282)
CLTA4 lib adapter2	TTG TAT CTC GTA TGC CGT CTT CTG CTT G (SEQ ID NO: 283)
CLTA1 sel PCR	CAA GCA GAA GAC GGC ATA CGA GAT TGT GTT CTC GGC AGG T (SEQ ID NO: 284)

TABLE 9-continued

oligonucleotide name	oligonucleotide sequence (5'->3')
CLTA2 sel PCR	CAA GCA GAA GAC GGC ATA CGA GAT AGA AGA CTC GGC AGG T (SEQ ID NO: 285)
CLTA3 sel PCR	CAA GCA GAA GAC GGC ATA CGA GAT TTC TCT CTC GGC AGG T (SEQ ID NO: 286)
CLTA4 sel PCR	CAA GCA GAA GAC GGC ATA CGA GAT ACA CAA CTC GGC AGG T (SEQ ID NO: 287)
PE2 short	AAT GAT ACG GCG ACC ACC GA (SEQ ID NO: 288)
CLTA1 lib seq PCR	AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TNN NNA CCT ACC TGC CGA GAA CAC A (SEQ ID NO: 289)
CLTA2 lib seq PCR	AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TNN NNA CCT ACC TGC CGA GTC TTC T (SEQ ID NO: 290)
CLTA3 lib seq PCR	AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TNN NNA CCT ACC TGC CGA GAG AGA A (SEQ ID NO: 291)
CLTA4 lib seq PCR	AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TNN NNA CCT ACC TGC CGA GTT GTG T (SEQ ID NO: 292)
lib fwd PCR	CAA GCA GAA GAC GGC ATA CGA GAT (SEQ ID NO: 293)
CLTA1-0-1 (Chr. 9) fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT CAA GTC TAG CAA GCA GGC CA (SEQ ID NO: 294)
CLTA1-0-1 (Chr. 12) fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT CAG GCA CTG AGT GGG AAA GT (SEQ ID NO: 295)
CLTA1-1-1 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT TAA CCC CAA GTC AGC AAG CA (SEQ ID NO: 296)
CLTA1-2-1 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT TTG CTG GTC AAT ACC CTG GC (SEQ ID NO: 297)
CLTA1-2-2 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT TGA GTA CCC CTG AAA TGG GC (SEQ ID NO: 298)
CLTA1-3-1 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT TCG CTA CCA ATC AGG GCT TT (SEQ ID NO: 299)
CLTA1-3-2 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT CCA TTG CCA CTT GTT TGC AT (SEQ ID NO: 300)
CLTA1-4-1 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT CCT ACC CCC ACA ACT TTG CT (SEQ ID NO: 301)
CLTA1-4-2 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT GTG TAC ATC CAG TGC ACC CA (SEQ ID NO: 302)
CLTA1-4-3 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT TCG GAA AGG ACT TTG AAT ACT TGT (SEQ ID NO: 303)
CLTA1-4-4 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT CGG CCC AAG ACC TCA TTC AC (SEQ ID NO: 304)
CLTA1-4-5 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT GTC CTC TCT GGG GCA GAA GT (SEQ ID NO: 305)
CLTA1-4-6 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT AGC TGA GTC ATG AGT TGT CTC C (SEQ ID NO: 306)
CLTA1-4-7 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT CTG CCA GCT TCT CAC ACC AT (SEQ ID NO: 307)
CLTA1-4-8 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT CTG AAG GAC AAA GGC GGG AA (SEQ ID NO: 308)

TABLE 9-continued

oligonucleotide name	oligonucleotide sequence (5'->3')
CLTA1-5-1 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT AAG GTG CTA AAG GCT CCA CG (SEQ ID NO: 309)
CLTA1-5-2 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT GAC CAT TGG TGA GCC CAG AG (SEQ ID NO: 310)
CLTA1-5-3 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT TTT TTC GGG CAA CTG CTC AC (SEQ ID NO: 311)
CLTA1-5-4 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT GCA AGC CTT CTC TCC TCA GA (SEQ ID NO: 312)
CLTA1-5-5 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT ACA CAA ACT TCC CTG AGA CCC (SEQ ID NO: 313)
CLTA1-6-1 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT TGA GTT AGC CCT GCT GTT CA (SEQ ID NO: 314)
CLTA4-0-1 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT TGA AGA GCT TCA CTG AGT AGG A (SEQ ID NO: 315)
CLTA4-3-1 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT TCC CCT TAC AGC CAA TTT CGT (SEQ ID NO: 316)
CLTA4-3-2 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT TGC TGA TGA AAT GCA ATT AAG AGG T (SEQ ID NO: 317)
CLTA4-3-3 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT GGT CCC TGC AAG CCA GTA TG (SEQ ID NO: 318)
CLTA4-3-4 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT ATC AAA GCC TTG TAT CAC AGT T (SEQ ID NO: 319)
CLTA4-3-5 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT CCC AAA TAA TGC AGG AGC CAA (SEQ ID NO: 320)
CLTA4-3-6 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT CTG CCT TTA GTG GGA CAG ACT T (SEQ ID NO: 321)
CLTA4-3-7 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT AGT AAC CCT AGT AGC CCT CCA (SEQ ID NO: 322)
CLTA4-4-1 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT CAT TGC AGT GAG CCG AGA TTG (SEQ ID NO: 323)
CLTA4-4-2 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT TGG CAA AGT TCA CTT CCA TGT (SEQ ID NO: 324)
CLTA4-4-3 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT TGC TCT GTG ATG TCT GCC AC (SEQ ID NO: 325)
CLTA4-4-4 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT TGT GTA GGA TTG TGA ACC AGC A (SEQ ID NO: 326)
CLTA4-4-5 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT TCC CAG CCC AGC ATT TTT CT (SEQ ID NO: 327)
CLTA4-4-6 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT AGG TTG CTT TGT GCA CAG TC (SEQ ID NO: 328)
CLTA4-4-7 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT CCT GGC TTG GGA TGT TGG AA (SEQ ID NO: 329)
CLTA4-4-8 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT TTG CCC AAG GTC ATA CTG CT (SEQ ID NO: 330)
CLTA4-4-9 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT ACC CAC TAG GTA GCC ATA ATC CA (SEQ ID NO: 331)
CLTA4-4-10 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT CGG TCA TGT CGC TTG GAA GA (SEQ ID NO: 332)
CLTA4-4-11 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT TTG GCC CAT ATT GCT TTA TGC TG (SEQ ID NO: 333)

TABLE 9-continued

oligonucleotide name	oligonucleotide sequence (5'->3')
CLTA4-4-12 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT ATT AGG GGT TGG CTG CAT GA (SEQ ID NO: 334)
CLTA4-4-13 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT CCA AGA CGT GTT GCA TGC TG (SEQ ID NO: 335)
CLTA4-4-14 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT TGG GAG GTG ATA AAT TCC CTA AAT (SEQ ID NO: 336)
CLTA4-5-1 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT CCA GAG ACA AAG GTG GGG AG (SEQ ID NO: 337)
CLTA4-5-2 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT TCA TAC AGA AGA GCA AAG TAC CA (SEQ ID NO: 338)
CLTA4-5-3 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT CAA AGA GGG GTA TCG GGA GC (SEQ ID NO: 339)
CLTA4-5-4 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT AAA TGG AAG AAC CAA GTA GAT GAA (SEQ ID NO: 340)
CLTA4-5-5 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT TTT TGG TTG ACA GAT GGC CAC A (SEQ ID NO: 341)
CLTA4-5-6 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT TCT TAC TTG TGT GAT TTT AGA ACA A (SEQ ID NO: 342)
CLTA4-6-1 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT GAT GGT TCA TGC AGA GGG CT (SEQ ID NO: 343)
CLTA4-6-2 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT GCT GGT CTT TCC TGA GCT GT (SEQ ID NO: 344)
CLTA4-6-3 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT CTC CAT CAG ATA CCT GTA CCC A (SEQ ID NO: 345)
CLTA4-7-1 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT GGG AAA ACA CTC TCT CTC TGC T (SEQ ID NO: 346)
CLTA4-7-2 fwd	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT GGA GGC CAC GAC ACA CAA TA (SEQ ID NO: 347)
CLTA1-0-1 (Chr. 9) rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT CAC AGG GTG GCT CTT CAG TG (SEQ ID NO: 348)
CLTA1-0-1 (Chr. 12) rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT TGC ACA TGT TTC CAC AGG GT (SEQ ID NO: 349)
CLTA1-1-1 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT AGT GTT TCC AGG AGC GGT TT (SEQ ID NO: 350)
CLTA1-2-1 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT AAG CCT CAG GCA CAA CTC TG (SEQ ID NO: 351)
CLTA1-2-2 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT TAG GGG AGG GGC AAA GAC A (SEQ ID NO: 352)
CLTA1-3-1 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT GGG AAC AGT GGT ATG CTG GT (SEQ ID NO: 353)
CLTA1-3-2 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT AGT GTG GAC ACT GAC AAG GAA (SEQ ID NO: 354)
CLTA1-4-1 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT TCA CTG CCT GGG TGC TTT AG (SEQ ID NO: 355)
CLTA1-4-2 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT TAC CCC AGC CTC CAG CTT TA (SEQ ID NO: 356)
CLTA1-4-3 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT TGA CTA CTG GGG AGC GAT GA (SEQ ID NO: 357)
CLTA1-4-4 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT AGG CTG TTA TGC AGG AAA GGA A (SEQ ID NO: 358)
CLTA1-4-5 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT GCG GTT GAG GTG GAT GGA AG (SEQ ID NO: 359)

TABLE 9-continued

oligonucleotide name	oligonucleotide sequence (5'->3')
CLTA1-4-6 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT GGC AGC ATC CCT TAC ATC CT (SEQ ID NO: 360)
CLTA1-4-7 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT AGA AAA AGC TTC CCC AGA AAG GA (SEQ ID NO: 361)
CLTA1-4-8 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT CTG CAC CAA CCT CTA CGT CC (SEQ ID NO: 362)
CLTA1-5-1 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT CTG GAG AGG GCA TAG TTG GC (SEQ ID NO: 363)
CLTA1-5-2 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT TGG AAG GCT CTT TGT GGG TT (SEQ ID NO: 364)
CLTA1-5-3 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT TTC CTA GCG GGA ACT GGA AA (SEQ ID NO: 365)
CLTA1-5-4 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT AGG CTA ATG GGG TAG GGG AT (SEQ ID NO: 366)
CLTA1-5-5 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT TGT CCA TGT TGG CTG AGG TG (SEQ ID NO: 367)
CLTA1-6-1 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT CAG GCC AAC CTT GAC AAC TT (SEQ ID NO: 368)
CLTA4-0-1 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT AGC AGG CCA AAG ATG TCT CC (SEQ ID NO: 369)
CLTA4-3-1 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT TCT GCT CTT GAG GTT ATT TGT CC (SEQ ID NO: 370)
CLTA4-3-2 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT GGG ACC AAT TTG CTA CTC ATG G (SEQ ID NO: 371)
CLTA4-3-3 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT TGG AGG CTG TAA ACG TCC TG (SEQ ID NO: 372)
CLTA4-3-4 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT TGC TAT GAT TTG CTG AAT TAC TCC T (SEQ ID NO: 373)
CLTA4-3-5 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT GCA ATT TTG CAG ACC ACC ATC (SEQ ID NO: 374)
CLTA4-3-6 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT GGC AGC TTG CAA CCT TCT TG (SEQ ID NO: 375)
CLTA4-3-7 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT TCA TGA GAG TTT CCC CAA CA (SEQ ID NO: 376)
CLTA4-4-1 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT ACT TGA GGG GGA AAA AGT TTC TTA (SEQ ID NO: 377)
CLTA4-4-2 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT TGG TCC CTG TCT GTC ATT GG (SEQ ID NO: 378)
CLTA4-4-3 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT AAG CGA GTG ACT GTC TGG GA (SEQ ID NO: 379)
CLTA4-4-4 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT CAT GGG TGG GAC ACG TAG TT (SEQ ID NO: 380)
CLTA4-4-5 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT GGC TTT CCT GGA CAC CCT ATC (SEQ ID NO: 381)
CLTA4-4-6 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT AGA GCG AGG GAG CGA TGT A (SEQ ID NO: 382)
CLTA4-4-7 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT TTG TGG ACC ACT GCT TAG TGC (SEQ ID NO: 383)
CLTA4-4-8 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT CAA CTA CCC TGA GGC CAC C (SEQ ID NO: 384)

TABLE 9-continued

oligonucleotide name	oligonucleotide sequence (5'->3')
CLTA4-4-9 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT GGT CAG CAC TCC TCA GCT TT (SEQ ID NO: 385)
CLTA4-4-10 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT TGG AGG ATG CAT GCC ACA TT (SEQ ID NO: 386)
CLTA4-4-11 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT CCC AGC CTC TTT GAC CCT TC (SEQ ID NO: 387)
CLTA4-4-12 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT CCC ACA CCA GGC TGT AAG G (SEQ ID NO: 388)
CLTA4-4-13 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT TAG ATA TAT GGG TGT GTC TGT ACG (SEQ ID NO: 389)
CLTA4-4-14 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT TTC CAA AGT GGC TGA ACC AT (SEQ ID NO: 390)
CLTA4-5-1 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT CCC ACA GGG CTG ATG TTT CA (SEQ ID NO: 391)
CLTA4-5-2 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT TTG TAA TGC AAC CTC TGT CAT GC (SEQ ID NO: 392)
CLTA4-5-3 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT CCA GCT CCA GCA ATC CAT GA (SEQ ID NO: 393)
CLTA4-5-4 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT TTT GGG AAA GAT AGC CCT GGA (SEQ ID NO: 394)
CLTA4-5-5 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT CAA TGA AAC AGC GGG GAG GT (SEQ ID NO: 395)
CLTA4-5-6 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT ACA ATC ACG TGT CCT TCA CT (SEQ ID NO: 396)
CLTA4-6-1 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT CAG ATC CCT CCT GGG CAA TG (SEQ ID NO: 397)
CLTA4-6-2 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT GTC AGG AGG CAA GGA GGA AC (SEQ ID NO: 398)
CLTA4-6-3 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT ACT TCC TTC CTT TTG AGA CCA AGT (SEQ ID NO: 399)
CLTA4-7-1 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT GCG GCA GAT TCC TGG TGA TT (SEQ ID NO: 400)
CLTA4-7-2 rev	GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATCT GGT CAC CAT CAG CAC AGT CA (SEQ ID NO: 401)
PE1-barcode1	CAA GCA GAA GAC GGC ATA CGA GAT ATA TCA GTG TGA CTG GAG TTC AGA CGT GTG CT (SEQ ID NO: 402)
PE1-barcode2	CAA GCA GAA GAC GGC ATA CGA GAT TTT CAC CGG TGA CTG GAG TTC AGA CGT GTG CT (SEQ ID NO: 403)
PE1-barcode3	CAA GCA GAA GAC GGC ATA CGA GAT CCA CTC ATG TGA CTG GAG TTC AGA CGT GTG CT (SEQ ID NO: 404)
PE1-barcode4	CAA GCA GAA GAC GGC ATA CGA GAT TAC GTA CGG TGA CTG GAG TTC AGA CGT GTG CT (SEQ ID NO: 405)
PE1-barcode5	CAA GCA GAA GAC GGC ATA CGA GAT CGA AAC TCG TGA CTG GAG TTC AGA CGT GTG CT (SEQ ID NO: 406)

TABLE 9-continued

oligonucleotide name	oligonucleotide sequence (5'->3')
PE1-barcode6	CAA GCA GAA GAC GGC ATA CGA GAT ATC AGT ATG TGA CTG GAG TTC AGA CGT GTG CT (SEQ ID NO: 407)
PE2-barcode1	AAT GAT ACG GCG ACC ACC GAG ATC TAC ACA TTA CTC GAC ACT CTT TCC CTA CAC GAC (SEQ ID NO: 408)
PE2-barcode2	AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CCG GAG AAC ACT CTT TCC CTA CAC GAC (SEQ ID NO: 409)
PE2-barcode3	AAT GAT ACG GCG ACC ACC GAG ATC TAC ACC GCT CAT TAC ACT CTT TCC CTA CAC GAC (SEQ ID NO: 410)

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All publications, patents and sequence database entries mentioned herein, including those items listed above, are hereby incorporated by reference in their entirety as if each individual publication or patent was specifically and individually indicated to be incorporated by reference. In case of conflict, the present application, including any definitions herein, will control.

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aaacgaatcg aagaaggtat caaagaatta ggaagtcaga ttcttaaaga gcatcctgtt 2400

gaaaatactc aattgcaaaa tgaaaagctc tatctctatt atctacaaaa tggaagagac 2460

atgtatgtgg accaagaatt agatattaat cgtttaagtg attatgatgt cgatcacatt 2520

gttccacaaa gtttcattaa agacgattca atagacaata aggtactaac gcgttctgat 2580

aaaaatcgtg gtaaatcgga taacgttcca agtgaagaag tagtcaaaaa gatgaaaaac 2640

tattggagac aacttctaaa cgccaagtta atcactcaac gtaagtttga taatttaacg 2700

aaagctgaac gtggaggttt gagtgaactt gataaagctg gttttatcaa acgccaattg 2760

gttgaaactc gccaaatcac taagcatgtg gcacaaattt tggatagtcg catgaatact 2820

aaatacgatg aaaatgataa acttattcga gaggttaaag tgattacctt aaaatctaaa 2880

ttagtttctg acttccgaaa agatttccaa ttctataaag tacgtgagat taacaattac 2940

catcatgccc atgatgcgta tctaaatgcc gtcgttggaa ctgctttgat taagaaatat 3000

ccaaaacttg aatcggagtt tgtctatggt gattataaag tttatgatgt tcgtaaaatg 3060

attgctaagt ctgagcaaga aataggcaaa gcaaccgcaa aatatttctt ttactctaat 3120

atcatgaact tcttcaaaac agaaattaca cttgcaaatg gagagattcg caaacgccct 3180

ctaatcgaaa ctaatgggga aactggagaa attgtctggg ataaagggcg agattttgcc 3240

acagtgcgca aagtattgtc catgccccaa gtcaatattg tcaagaaaac agaagtacag 3300

acaggcggat tctccaagga gtcaatttta ccaaaaagaa attcggacaa gcttattgct 3360

cgtaaaaaag actgggatcc aaaaaaatat ggtggttttg atagtccaac ggtagcttat 3420

tcagtcctag tggttgctaa ggtggaaaaa gggaaatcga agaagttaaa atccgttaaa 3480

gagttactag ggatcacaat tatggaaaga agttcctttg aaaaaaatcc gattgacttt 3540

ttagaagcta aaggatataa ggaagttaaa aaagacttaa tcattaaact acctaaatat 3600

agtctttttg agttagaaaa cggtcgtaaa cggatgctgg ctagtgccgg agaattacaa 3660

aaaggaaatg agctggctct gccaagcaaa tatgtgaatt ttttatattt agctagtcat 3720

tatgaaaagt tgaagggtag tccagaagat aacgaacaaa aacaattgtt tgtggagcag 3780

cataagcatt atttagatga gattattgag caaatcagtg aattttctaa gcgtgttatt 3840

ttagcagatg ccaatttaga taaagttctt agtgcatata acaaacatag agacaaacca 3900

atacgtgaac aagcagaaaa tattattcat ttatttacgt tgacgaatct tggagctccc 3960

gctgctttta aatattttga tacaacaatt gatcgtaaac gatatacgtc tacaaaagaa 4020

gttttagatg ccactcttat ccatcaatcc atcactggtc tttatgaaac acgcattgat 4080

ttgagtcagc taggaggtga ctga 4104

<210> SEQ ID NO 41
<211> LENGTH: 1367
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Polypeptide

<400> SEQUENCE: 41

Met Asp Lys Lys Tyr Ser Ile Gly Leu Asp Ile Gly Thr Asn Ser Val
1 5 10 15

Gly Trp Ala Val Ile Thr Asp Asp Tyr Lys Val Pro Ser Lys Lys Phe
20 25 30

-continued

```
Lys Val Leu Gly Asn Thr Asp Arg His Ser Ile Lys Lys Asn Leu Ile
        35                  40                  45

Gly Ala Leu Leu Phe Gly Ser Gly Glu Thr Ala Glu Ala Thr Arg Leu
    50                  55                  60

Lys Arg Thr Ala Arg Arg Arg Tyr Thr Arg Arg Lys Asn Arg Ile Cys
65                  70                  75                  80

Tyr Leu Gln Glu Ile Phe Ser Asn Glu Met Ala Lys Val Asp Asp Ser
                85                  90                  95

Phe Phe His Arg Leu Glu Glu Ser Phe Leu Val Glu Glu Asp Lys Lys
            100                 105                 110

His Glu Arg His Pro Ile Phe Gly Asn Ile Val Asp Glu Val Ala Tyr
        115                 120                 125

His Glu Lys Tyr Pro Thr Ile Tyr His Leu Arg Lys Lys Leu Ala Asp
    130                 135                 140

Ser Thr Asp Lys Ala Asp Leu Arg Leu Ile Tyr Leu Ala Leu Ala His
145                 150                 155                 160

Met Ile Lys Phe Arg Gly His Phe Leu Ile Glu Gly Asp Leu Asn Pro
                165                 170                 175

Asp Asn Ser Asp Val Asp Lys Leu Phe Ile Gln Leu Val Gln Ile Tyr
            180                 185                 190

Asn Gln Leu Phe Glu Glu Asn Pro Ile Asn Ala Ser Arg Val Asp Ala
        195                 200                 205

Lys Ala Ile Leu Ser Ala Arg Leu Ser Lys Ser Arg Arg Leu Glu Asn
    210                 215                 220

Leu Ile Ala Gln Leu Pro Gly Glu Lys Arg Asn Gly Leu Phe Gly Asn
225                 230                 235                 240

Leu Ile Ala Leu Ser Leu Gly Leu Thr Pro Asn Phe Lys Ser Asn Phe
                245                 250                 255

Asp Leu Ala Glu Asp Ala Lys Leu Gln Leu Ser Lys Asp Thr Tyr Asp
            260                 265                 270

Asp Asp Leu Asp Asn Leu Leu Ala Gln Ile Gly Asp Gln Tyr Ala Asp
        275                 280                 285

Leu Phe Leu Ala Ala Lys Asn Leu Ser Asp Ala Ile Leu Leu Ser Asp
    290                 295                 300

Ile Leu Arg Val Asn Ser Glu Ile Thr Lys Ala Pro Leu Ser Ala Ser
305                 310                 315                 320

Met Ile Lys Arg Tyr Asp Glu His His Gln Asp Leu Thr Leu Leu Lys
                325                 330                 335

Ala Leu Val Arg Gln Gln Leu Pro Glu Lys Tyr Lys Glu Ile Phe Phe
            340                 345                 350

Asp Gln Ser Lys Asn Gly Tyr Ala Gly Tyr Ile Asp Gly Gly Ala Ser
        355                 360                 365

Gln Glu Glu Phe Tyr Lys Phe Ile Lys Pro Ile Leu Glu Lys Met Asp
    370                 375                 380

Gly Thr Glu Glu Leu Leu Val Lys Leu Asn Arg Glu Asp Leu Leu Arg
385                 390                 395                 400

Lys Gln Arg Thr Phe Asp Asn Gly Ser Ile Pro His Gln Ile His Leu
                405                 410                 415

Gly Glu Leu His Ala Ile Leu Arg Arg Gln Glu Asp Phe Tyr Pro Phe
            420                 425                 430

Leu Lys Asp Asn Arg Glu Lys Ile Glu Lys Ile Leu Thr Phe Arg Ile
        435                 440                 445
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Pro Tyr Tyr Val Gly Pro Leu Ala Arg Gly Asn Ser Arg Phe Ala Trp
    450                 455                 460

Met Thr Arg Lys Ser Glu Glu Thr Ile Thr Pro Trp Asn Phe Glu Glu
465                 470                 475                 480

Val Val Asp Lys Gly Ala Ser Ala Gln Ser Phe Ile Glu Arg Met Thr
                485                 490                 495

Asn Phe Asp Lys Asn Leu Pro Asn Glu Lys Val Leu Pro Lys His Ser
            500                 505                 510

Leu Leu Tyr Glu Tyr Phe Thr Val Tyr Asn Glu Leu Thr Lys Val Lys
        515                 520                 525

Tyr Val Thr Glu Gly Met Arg Lys Pro Ala Phe Leu Ser Gly Glu Gln
    530                 535                 540

Lys Lys Ala Ile Val Asp Leu Leu Phe Lys Thr Asn Arg Lys Val Thr
545                 550                 555                 560

Val Lys Gln Leu Lys Glu Asp Tyr Phe Lys Lys Ile Glu Cys Phe Asp
                565                 570                 575

Ser Val Glu Ile Ser Gly Val Glu Asp Arg Phe Asn Ala Ser Leu Gly
            580                 585                 590

Ala Tyr His Asp Leu Leu Lys Ile Ile Lys Asp Lys Asp Phe Leu Asp
        595                 600                 605

Asn Glu Glu Asn Glu Asp Ile Leu Glu Asp Ile Val Leu Thr Leu Thr
    610                 615                 620

Leu Phe Glu Asp Arg Gly Met Ile Glu Glu Arg Leu Lys Thr Tyr Ala
625                 630                 635                 640

His Leu Phe Asp Asp Lys Val Met Lys Gln Leu Lys Arg Arg Arg Tyr
                645                 650                 655

Thr Gly Trp Gly Arg Leu Ser Arg Lys Leu Ile Asn Gly Ile Arg Asp
            660                 665                 670

Lys Gln Ser Gly Lys Thr Ile Leu Asp Phe Leu Lys Ser Asp Gly Phe
        675                 680                 685

Ala Asn Arg Asn Phe Met Gln Leu Ile His Asp Asp Ser Leu Thr Phe
    690                 695                 700

Lys Glu Asp Ile Gln Lys Ala Gln Val Ser Gly Gln Gly His Ser Leu
705                 710                 715                 720

His Glu Gln Ile Ala Asn Leu Ala Gly Ser Pro Ala Ile Lys Lys Gly
                725                 730                 735

Ile Leu Gln Thr Val Lys Ile Val Asp Glu Leu Val Lys Val Met Gly
            740                 745                 750

His Lys Pro Glu Asn Ile Val Ile Glu Met Ala Arg Glu Asn Gln Thr
        755                 760                 765

Thr Gln Lys Gly Gln Lys Asn Ser Arg Glu Arg Met Lys Arg Ile Glu
    770                 775                 780

Glu Gly Ile Lys Glu Leu Gly Ser Gln Ile Leu Lys Glu His Pro Val
785                 790                 795                 800

Glu Asn Thr Gln Leu Gln Asn Glu Lys Leu Tyr Leu Tyr Tyr Leu Gln
                805                 810                 815

Asn Gly Arg Asp Met Tyr Val Asp Gln Glu Leu Asp Ile Asn Arg Leu
            820                 825                 830

Ser Asp Tyr Asp Val Asp His Ile Val Pro Gln Ser Phe Ile Lys Asp
        835                 840                 845

Asp Ser Ile Asp Asn Lys Val Leu Thr Arg Ser Asp Lys Asn Arg Gly
    850                 855                 860
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Lys Ser Asp Asn Val Pro Ser Glu Glu Val Val Lys Lys Met Lys Asn
865                 870                 875                 880

Tyr Trp Arg Gln Leu Leu Asn Ala Lys Leu Ile Thr Gln Arg Lys Phe
                885                 890                 895

Asp Asn Leu Thr Lys Ala Glu Arg Gly Gly Leu Ser Glu Leu Asp Lys
            900                 905                 910

Ala Gly Phe Ile Lys Arg Gln Leu Val Glu Thr Arg Gln Ile Thr Lys
        915                 920                 925

His Val Ala Gln Ile Leu Asp Ser Arg Met Asn Thr Lys Tyr Asp Glu
    930                 935                 940

Asn Asp Lys Leu Ile Arg Glu Val Lys Val Ile Thr Leu Lys Ser Lys
945                 950                 955                 960

Leu Val Ser Asp Phe Arg Lys Asp Phe Gln Phe Tyr Lys Val Arg Glu
                965                 970                 975

Ile Asn Asn Tyr His His Ala His Asp Ala Tyr Leu Asn Ala Val Val
            980                 985                 990

Gly Thr Ala Leu Ile Lys Lys Tyr  Pro Lys Leu Glu Ser  Glu Phe Val
        995                 1000                1005

Tyr Gly  Asp Tyr Lys Val Tyr  Asp Val Arg Lys Met  Ile Ala Lys
    1010                1015                1020

Ser Glu  Gln Glu Ile Gly Lys  Ala Thr Ala Lys Tyr  Phe Phe Tyr
    1025                1030                1035

Ser Asn  Ile Met Asn Phe Phe  Lys Thr Glu Ile Thr  Leu Ala Asn
    1040                1045                1050

Gly Glu  Ile Arg Lys Arg Pro  Leu Ile Glu Thr Asn  Gly Glu Thr
    1055                1060                1065

Gly Glu  Ile Val Trp Asp Lys  Gly Arg Asp Phe Ala  Thr Val Arg
    1070                1075                1080

Lys Val  Leu Ser Met Pro Gln  Val Asn Ile Val Lys  Lys Thr Glu
    1085                1090                1095

Val Gln  Thr Gly Gly Phe Ser  Lys Glu Ser Ile Leu  Pro Lys Arg
    1100                1105                1110

Asn Ser  Asp Lys Leu Ile Ala  Arg Lys Lys Asp Trp  Asp Pro Lys
    1115                1120                1125

Lys Tyr  Gly Gly Phe Asp Ser  Pro Thr Val Ala Tyr  Ser Val Leu
    1130                1135                1140

Val Val  Ala Lys Val Glu Lys  Gly Lys Ser Lys Lys  Leu Lys Ser
    1145                1150                1155

Val Lys  Glu Leu Leu Gly Ile  Thr Ile Met Glu Arg  Ser Ser Phe
    1160                1165                1170

Glu Lys  Asn Pro Ile Asp Phe  Leu Glu Ala Lys Gly  Tyr Lys Glu
    1175                1180                1185

Val Lys  Lys Asp Leu Ile Ile  Lys Leu Pro Lys Tyr  Ser Leu Phe
    1190                1195                1200

Glu Leu  Glu Asn Gly Arg Lys  Arg Met Leu Ala Ser  Ala Gly Glu
    1205                1210                1215

Leu Gln  Lys Gly Asn Glu Leu  Ala Leu Pro Ser Lys  Tyr Val Asn
    1220                1225                1230

Phe Leu  Tyr Leu Ala Ser His  Tyr Glu Lys Leu Lys  Gly Ser Pro
    1235                1240                1245

Glu Asp  Asn Glu Gln Lys Gln  Leu Phe Val Glu Gln  His Lys His
    1250                1255                1260
```

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Tyr Leu  Asp Glu Ile Ile Glu  Gln Ile Ser Glu Phe  Ser Lys Arg
    1265                1270                1275

Val Ile  Leu Ala Asp Ala Asn  Leu Asp Lys Val Leu  Ser Ala Tyr
    1280                1285                1290

Asn Lys  His Arg Asp Lys Pro  Ile Arg Glu Gln Ala  Glu Asn Ile
    1295                1300                1305

Ile His  Leu Phe Thr Leu Thr  Asn Leu Gly Ala Pro  Ala Ala Phe
    1310                1315                1320

Lys Tyr  Phe Asp Thr Thr Ile  Asp Arg Lys Arg Tyr  Thr Ser Thr
    1325                1330                1335

Lys Glu  Val Leu Asp Ala Thr  Leu Ile His Gln Ser  Ile Thr Gly
    1340                1345                1350

Leu Tyr  Glu Thr Arg Ile Asp  Leu Ser Gln Leu Gly  Gly Asp
    1355                1360                1365


<210> SEQ ID NO 42
<211> LENGTH: 100
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 42

aacacatggg tcgacacaaa cacaactcgg caggtacttg cagatgtagt ctttccacat     60

gggtcgacac aaacacaact cggcaggtat ctcgtatgcc                          100


<210> SEQ ID NO 43
<211> LENGTH: 10
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 43

ctcggcaggt                                                            10


<210> SEQ ID NO 44
<211> LENGTH: 43
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 44

acttgcagat gtagtctttc cacatgggtc gacacaaaca caa                       43


<210> SEQ ID NO 45
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 45

tgtgtttgtg tt                                                         12


<210> SEQ ID NO 46
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide
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<400> SEQUENCE: 46

agaagaagaa ga 12

<210> SEQ ID NO 47
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 47

ttctctttct ct 12

<210> SEQ ID NO 48
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 48

acacaaacac aa 12

<210> SEQ ID NO 49
<211> LENGTH: 43
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 49

acttgcagat gtagtctttc cacatgggtc gacacaaaca caa 43

<210> SEQ ID NO 50
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 50

acttgcagat gtagtctttc cacatgggtc g 31

<210> SEQ ID NO 51
<211> LENGTH: 100
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 51

aacacatggg tcgacacaaa cacaactcgg caggtacttg cagatgtagt ctttccacat 60

gggtcgacac aaacacaact cggcaggtat ctcgtatgcc 100

<210> SEQ ID NO 52
<211> LENGTH: 162
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 52

caatctcccg catgcgctca gtcctcatct ccctcaagca ggccccgctg gtgcactgaa 60

gagccaccct gtgaaacact acatctgcaa tatcttaatc ctactcagtg aagctcttca 120

cagtcattgg attaattatg ttgagttctt ttggaccaaa cc 162

<210> SEQ ID NO 53
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 53

gctggtgcac tgaagagcca 20

<210> SEQ ID NO 54
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 54

aatatcttaa tcctactcag 20

<210> SEQ ID NO 55
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 55

ccctgtgaaa cactacatct gc 22

<210> SEQ ID NO 56
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 56

gcagatgtag tgtttcacag gg 22

<210> SEQ ID NO 57
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 57

gcagatgtag tgtttccaca ggg 23

<210> SEQ ID NO 58
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 58

gcagatgtag tgtttccaca ggg 23

<210> SEQ ID NO 59
<211> LENGTH: 23
<212> TYPE: DNA

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 59

acatatgtag tatttccaca ggg 23

<210> SEQ ID NO 60
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 60

gcatatgtag tgtttccaaa tgt 23

<210> SEQ ID NO 61
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 61

ccagatgtag tattcccaca ggg 23

<210> SEQ ID NO 62
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 62

gcagttttag tgttttcaca ggg 23

<210> SEQ ID NO 63
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 63

gcagagttag tgtttccaca cag 23

<210> SEQ ID NO 64
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 64

gcagatggag ggttttcaca ggg 23

<210> SEQ ID NO 65
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 65

ggaaatttag tgtttccaca ggg 23

<210> SEQ ID NO 66
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 66

aaagaagtag tatttccaca tgg 23

<210> SEQ ID NO 67
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 67

aaagatgtag tcattccaca agg 23

<210> SEQ ID NO 68
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 68

aaatatgtag tctttccaca ggg 23

<210> SEQ ID NO 69
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 69

atagatgtag tgtttccaaa gga 23

<210> SEQ ID NO 70
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 70

ccagaggtag tgctcccaca ggg 23

<210> SEQ ID NO 71
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 71

ccagatgtga ggtttccaca agg 23

<210> SEQ ID NO 72
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:

<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 72

ctacatgtag tgtttccata tgg 23

<210> SEQ ID NO 73
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 73

tagatgaagt gcttccacat gg 22

<210> SEQ ID NO 74
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 74

gaaaatggag tgtttacaca tgg 23

<210> SEQ ID NO 75
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 75

gcaaatgaag tgtcaccaca agg 23

<210> SEQ ID NO 76
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 76

gcaaatgtat tatttccact agg 23

<210> SEQ ID NO 77
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 77

gcagatgtag cttttgtaca tgg 23

<210> SEQ ID NO 78
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 78

gcagcttaag tgttttcaca tgg 23

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<210> SEQ ID NO 79
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 79

ttacatgtag tgtttacaca cgg 23

<210> SEQ ID NO 80
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 80

gaagaggaag tgtttgccca ggg 23

<210> SEQ ID NO 81
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 81

gaagatgtgg agttgacaca tgg 23

<210> SEQ ID NO 82
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 82

gcagaagtac tgttgttaca agg 23

<210> SEQ ID NO 83
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 83

gcagatgtgg aattacaaca ggg 23

<210> SEQ ID NO 84
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 84

gcagtcatag tgtatacaca tgg 23

<210> SEQ ID NO 85
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 85

taagatgtag tatttccaaa agt 23

<210> SEQ ID NO 86
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 86

gcagctggca tttctccaca cgg 23

<210> SEQ ID NO 87
<211> LENGTH: 23
<212> TYPE: DNA
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ggagatctga tggttctaca agg 23

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taaaatgcag tgtatccata tgg 23

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gccagaatag tttttcaaca agg 23

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ttgtatttag agattgcaca agg 23

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agtcctcatc tccctcaagc agg 23

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agtcctcaac tccctcaagc agg 23

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agccctcatt tccctcaagc agg 23

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actcctcatc cccctcaagc cgg 23

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agtcatcatc tccctcaagc aga 23

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cgtcctcctc tcccccaagc agg 23

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tgtcctcttc tccctcaagc aga 23

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aagcttcatc tctctcaagc tgg 23

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agtactcttt tccctcaggc tgg 23

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agtcttaaat tccctcaagc agg 23

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agtgctcatc taccagaagc tgg 23

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cctcctcatc tccctgcagc agg 23

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ctacatcatc tccctcaagc tgg 23

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ggtcctcatc tccctaaaac aga 23

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tgtcctcatc ggcctcaggc agg 23

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agacaccatc tcccttgagc tgg 23

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aggcatcatc tacatcaagt tgg 23

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agtaatcact tccatcaagc cgg 23

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tcccctcacc tccctaaagc agg 23

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tgtctttatt tccctctagc tgg 23

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agtcctcatc tccctcaagc agg 23

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<400> SEQUENCE: 112

gcagatgtag tgtttccaca ggg 23

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<400> SEQUENCE: 113

acatatgtag tatttccaca ggg 23

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<400> SEQUENCE: 114

gcatatgtag tgtttccaaa tgt 23

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<400> SEQUENCE: 115

ccagatgtag tattcccaca ggg 23

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<400> SEQUENCE: 116

gcagttttag tgttttcaca ggg 23

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<400> SEQUENCE: 117

gcagagttag tgtttccaca cag 23

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<400> SEQUENCE: 118

gcagatggag ggttttcaca ggg 23

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<400> SEQUENCE: 119

ggaaatttag tgtttccaca ggg 23

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<400> SEQUENCE: 120

aaagaagtag tatttccaca tgg 23

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<400> SEQUENCE: 121

aaagatgtag tcattccaca agg 23

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<400> SEQUENCE: 122

aaatatgtag tctttccaca ggg 23

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<400> SEQUENCE: 123

atagatgtag tgtttccaaa gga 23

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<400> SEQUENCE: 124

ccagaggtag tgctcccaca ggg 23

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<400> SEQUENCE: 125

ccagatgtga ggtttccaca agg 23

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<400> SEQUENCE: 126

ctacatgtag tgtttccata tgg 23

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<400> SEQUENCE: 127

ctagatgaag tgcttccaca tgg 23

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<400> SEQUENCE: 128

gaaaatggag tgtttacaca tgg 23

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<400> SEQUENCE: 129

gcaaatgaag tgtcaccaca agg 23

<210> SEQ ID NO 130
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<212> TYPE: DNA
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 130

gcaaatgtat tatttccact agg 23

<210> SEQ ID NO 131
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<400> SEQUENCE: 131

gcagatgtag cttttgtaca tgg 23

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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 132

gcagcttaag tgttttcaca tgg 23

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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 133

ttacatgtag tgtttacaca cgg 23

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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 134

gaagaggaag tgtttgccca ggg 23

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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 135

gaagatgtgg agttgacaca tgg 23

<210> SEQ ID NO 136
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 136

gcagaagtac tgttgttaca agg 23

<210> SEQ ID NO 137
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<212> TYPE: DNA
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 137

gcagatgtgg aattacaaca ggg 23

<210> SEQ ID NO 138
<211> LENGTH: 23
<212> TYPE: DNA

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<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 138

gcagtcatag tgtatacaca tgg 23

<210> SEQ ID NO 139
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 139

taagatgtag tatttccaaa agt 23

<210> SEQ ID NO 140
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<400> SEQUENCE: 140

gcagctggca tttctccaca cgg 23

<210> SEQ ID NO 141
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 141

ggagatctga tggttctaca agg 23

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<212> TYPE: DNA
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 142

taaaatgcag tgtatccata tgg 23

<210> SEQ ID NO 143
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 143

gccagaatag tttttcaaca agg 23

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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 144

ttgtatttag agattgcaca agg 23

<210> SEQ ID NO 145
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 145

agtcctcatc tccctcaagc agg 23

<210> SEQ ID NO 146
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<400> SEQUENCE: 146

agtcctcaac tccctcaagc agg 23

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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 147

agccctcatt tccctcaagc agg 23

<210> SEQ ID NO 148
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 148

actcctcatc cccctcaagc cgg 23

<210> SEQ ID NO 149
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 149

agtcatcatc tccctcaagc aga 23

<210> SEQ ID NO 150
<211> LENGTH: 23
<212> TYPE: DNA
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 150

cgtcctcctc tcccccaagc agg 23

<210> SEQ ID NO 151
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 151

tgtcctcttc tccctcaagc aga 23

<210> SEQ ID NO 152
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 152

aagcttcatc tctctcaagc tgg 23

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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 153

agtactcttt tccctcaggc tgg 23

<210> SEQ ID NO 154
<211> LENGTH: 23
<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 154

agtcttaaat tccctcaagc agg 23

<210> SEQ ID NO 155
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 155

agtgctcatc taccagaagc tgg 23

<210> SEQ ID NO 156
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<212> TYPE: DNA
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 156

cctcctcatc tccctgcagc agg 23

<210> SEQ ID NO 157
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 157

ctacatcatc tccctcaagc tgg 23

<210> SEQ ID NO 158
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 158

ggtcctcatc tccctaaaac aga 23

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<212> TYPE: DNA
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 159

tgtcctcatc ggcctcaggc agg 23

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<211> LENGTH: 23
<212> TYPE: DNA
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<400> SEQUENCE: 160

agacaccatc tcccttgagc tgg 23

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<400> SEQUENCE: 161

aggcatcatc tacatcaagt tgg 23

<210> SEQ ID NO 162
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<212> TYPE: DNA
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<400> SEQUENCE: 162

agtaatcact tccatcaagc cgg 23

<210> SEQ ID NO 163
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 163

tcccctcacc tccctaaagc agg 23

<210> SEQ ID NO 164
<211> LENGTH: 23
<212> TYPE: DNA
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

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<400> SEQUENCE: 164

tgtctttatt tccctctagc tgg 23

<210> SEQ ID NO 165
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<212> TYPE: DNA
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 165

agtcctcatc tccctcaagc agg 23

<210> SEQ ID NO 166
<211> LENGTH: 23
<212> TYPE: DNA
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 166

agtcctcatc tccctcaagc agg 23

<210> SEQ ID NO 167
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 167

agtcctcatc tccctcaaag cagg 24

<210> SEQ ID NO 168
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 168

agtcctcatc tccctcagca gg 22

<210> SEQ ID NO 169
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 169

agtcctcatc tccctcatag cagg 24

<210> SEQ ID NO 170
<211> LENGTH: 15
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 170

agtcctcata gcagg 15

<210> SEQ ID NO 171
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<212> TYPE: DNA
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 171

agtcctcatc tagcagg 17

<210> SEQ ID NO 172
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 172

agtcctcaag cagg 14

<210> SEQ ID NO 173
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 173

agtcctcatc tccctcaaag gcagtgtttg ttacttgagt ttgtcagcag g 51

<210> SEQ ID NO 174
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 174

agtcctcatc tccctcatta gcagg 25

<210> SEQ ID NO 175
<211> LENGTH: 65
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 175

agtcctcatc tccctcaggg cttgtttaca gctcaccttt gaatttgcac aagcgtgcaa 60

gcagg 65

<210> SEQ ID NO 176
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 176

agtcctcatc tccctagcag g 21

<210> SEQ ID NO 177
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

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<400> SEQUENCE: 177

agtcctcatc cctcaagcag g 21

<210> SEQ ID NO 178
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 178

agtcctcatc tccctaagca gg 22

<210> SEQ ID NO 179
<211> LENGTH: 23
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<400> SEQUENCE: 179

agtcctcaac tccctcaagc agg 23

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<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 180

agtcctcaac tccctcaaag cagg 24

<210> SEQ ID NO 181
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<400> SEQUENCE: 181

agtcctcaac tccctca 17

<210> SEQ ID NO 182
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<212> TYPE: DNA
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<400> SEQUENCE: 182

agtcctcaac tccctcagca gg 22

<210> SEQ ID NO 183
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 183

agtcctcaac tccctcaaga aaggtgttga aaatcagaaa gagagaaaca agcagg 56

<210> SEQ ID NO 184
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 184

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gg 62

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agtcctcaac tccctaagca gg 22

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<400> SEQUENCE: 186

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agcagg 66

<210> SEQ ID NO 187
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<212> TYPE: DNA
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<400> SEQUENCE: 187

agtcctcaac tccctcacag caaataaaaa agttgtttat gcatattcag ataagcaaag 60

cagg 64

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<400> SEQUENCE: 188

agtcctcaac tcccaagcag g 21

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<400> SEQUENCE: 189

actcctcatc cccctcaagc cgg 23

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<400> SEQUENCE: 190

actcctcatc cccctcaaag ccgg 24

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<400> SEQUENCE: 191

actcctcatc cccctcagag ccgg 24

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<400> SEQUENCE: 192

actcctcagc cgg 13

<210> SEQ ID NO 193
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<400> SEQUENCE: 193

actcctcatc cccctcaaaa gccgg 25

<210> SEQ ID NO 194
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<400> SEQUENCE: 194

actcctcatc cccctcagca gccgg 25

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<400> SEQUENCE: 195

actcctcatc cccctcatag ccgg 24

<210> SEQ ID NO 196
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<212> TYPE: DNA
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<400> SEQUENCE: 196

actcctcatc cccctcatcc ccgg 24

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<400> SEQUENCE: 197

actcctcatc ccagccgg 18

<210> SEQ ID NO 198
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<400> SEQUENCE: 198

actcctcatc cccctaagcc gg 22

<210> SEQ ID NO 199
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<212> TYPE: DNA
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<400> SEQUENCE: 199

actcctcatc cccctcaata gccgg 25

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<400> SEQUENCE: 200

actcctcacc cccctcagca gccgg 25

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<400> SEQUENCE: 201

gcagatgtag tgtttccaca ggg 23

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<400> SEQUENCE: 202

gcagatgtag tgtttcacag gg 22

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<220> FEATURE:

<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 203

gcagatgtag tgtttcccac aggg 24

<210> SEQ ID NO 204
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 204

gcagatgtag tgcacaggg 19

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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 205

gcagatgtag tgtttcccag gg 22

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<400> SEQUENCE: 206

gcagatgtac acaggg 16

<210> SEQ ID NO 207
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<212> TYPE: DNA
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 207

gcagatgtag tgtcacaggg 20

<210> SEQ ID NO 208
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 208

gcagatgtag tgttcacagg g 21

<210> SEQ ID NO 209
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 209

gcagatgtag tgtttccagg g 21

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<210> SEQ ID NO 210
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 210

gcagatgtag tcacaggg 18

<210> SEQ ID NO 211
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<212> TYPE: DNA
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<400> SEQUENCE: 211

gcagatgtag gg 12

<210> SEQ ID NO 212
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 212

acatatgtag tatttccaca ggg 23

<210> SEQ ID NO 213
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<212> TYPE: DNA
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 213

acatatgtag tatttcccac aggg 24

<210> SEQ ID NO 214
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 214

acatatgtag tatttcacag gg 22

<210> SEQ ID NO 215
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<212> TYPE: DNA
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 215

acatatgtag tatttccagg g 21

<210> SEQ ID NO 216
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<400> SEQUENCE: 216

acatatcaca ggg 13

<210> SEQ ID NO 217
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<212> TYPE: DNA
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 217

acatcacagg g 11

<210> SEQ ID NO 218
<211> LENGTH: 18
<212> TYPE: DNA
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 218

acatatgtag tcacaggg 18

<210> SEQ ID NO 219
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<212> TYPE: DNA
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<220> FEATURE:
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<400> SEQUENCE: 219

acatatgtag tatttcc 17

<210> SEQ ID NO 220
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 220

acatatgtag gg 12

<210> SEQ ID NO 221
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<220> FEATURE:
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<400> SEQUENCE: 221

acatatgtag cacaggg 17

<210> SEQ ID NO 222
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 222

ccagatgtag tattcccaca ggg 23

<210> SEQ ID NO 223
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 223

ccagatgtag tattccccac aggg 24

<210> SEQ ID NO 224
<211> LENGTH: 19
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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 224

ccagatgtag tatacaggg 19

<210> SEQ ID NO 225
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 225

ccagatgtag tatcacaggg 20

<210> SEQ ID NO 226
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 226

ccagatgtag tattcccaac acaggg 26

<210> SEQ ID NO 227
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 227

ccagatgtag tattcacagg g 21

<210> SEQ ID NO 228
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 228

ccagatgtag tattcccagg g 21

<210> SEQ ID NO 229
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 229

ccagatgtag tattccacag gg 22

<210> SEQ ID NO 230
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 230

ctagatgaag tgcttccaca tgg 23

<210> SEQ ID NO 231
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 231

ctagatgaag tgcttcccac atgg 24

<210> SEQ ID NO 232
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 232

ctagatgaag tgcttcacat gg 22

<210> SEQ ID NO 233
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 233

ctagatgaag tg 12

<210> SEQ ID NO 234
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 234

ctagatgaag tgcttccaca catgg 25

<210> SEQ ID NO 235
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 235

ctagatgaag tgcttccatg g 21

<210> SEQ ID NO 236
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence

<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 236

ctagatgaag tgcttcccat gg 22

<210> SEQ ID NO 237
<211> LENGTH: 56
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 237

taatacgact cactatagga gtcctcatct ccctcaagcg ttttagagct atgctg 56

<210> SEQ ID NO 238
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 238

taatacgact cactataggc tccctcaagc aggccccgcg ttttagagct atgctg 56

<210> SEQ ID NO 239
<211> LENGTH: 56
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 239

taatacgact cactataggt gtgaagagct tcactgagtg ttttagagct atgctg 56

<210> SEQ ID NO 240
<211> LENGTH: 56
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 240

taatacgact cactataggg cagatgtagt gtttccacag ttttagagct atgctg 56

<210> SEQ ID NO 241
<211> LENGTH: 55
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 241

gataacggac tagccttatt ttaacttgct atgcttttca gcatagctct aaaac 55

<210> SEQ ID NO 242
<211> LENGTH: 81
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 242

cggactagcc ttattttaac ttgctatttc tagctctaaa acgcttgagg gagatgagga 60

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ctcctatagt gagtcgtatt a 81

<210> SEQ ID NO 243
<211> LENGTH: 81
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 243

cggactagcc ttattttaac ttgctatttc tagctctaaa acgcggggcc tgcttgaggg 60

agcctatagt gagtcgtatt a 81

<210> SEQ ID NO 244
<211> LENGTH: 81
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 244

cggactagcc ttattttaac ttgctatttc tagctctaaa acactcagtg aagctcttca 60

cacctatagt gagtcgtatt a 81

<210> SEQ ID NO 245
<211> LENGTH: 81
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 245

cggactagcc ttattttaac ttgctatttc tagctctaaa actgtggaaa cactacatct 60

gccctatagt gagtcgtatt a 81

<210> SEQ ID NO 246
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 246

taatacgact cactatagg 19

<210> SEQ ID NO 247
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Synthetic Oligonucleotide
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<222> LOCATION: (7)..(10)
<223> OTHER INFORMATION: n is a, c, g, or t
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<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (34)..(37)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 247

aacacannnn ccngcttgag ggagatgagg actnnnnacc tgccgagaac aca 53

<210> SEQ ID NO 248
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Synthetic Oligonucleotide
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<221> NAME/KEY: misc_feature
<222> LOCATION: (7)..(10)
<223> OTHER INFORMATION: n is a, c, g, or t
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<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (34)..(37)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 248

tcttctnnnn ccngcggggc ctgcttgagg gagnnnnacc tgccgagtct tct 53

<210> SEQ ID NO 249
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide
<220> FEATURE:
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<222> LOCATION: (7)..(10)
<223> OTHER INFORMATION: n is a, c, g, or t
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<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (34)..(37)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 249

agagaannnn ccnactcagt gaagctcttc acannnnacc tgccgagaga gaa 53

<210> SEQ ID NO 250
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide
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<222> LOCATION: (7)..(10)
<223> OTHER INFORMATION: n is a, c, g, or t
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<221> NAME/KEY: misc_feature
<222> LOCATION: (13)..(13)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (34)..(37)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 250

ttgtgtnnnn ccntgtggaa acactacatc tgcnnnnacc tgccgagttg tgt 53

<210> SEQ ID NO 251
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:

<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 251

ctagcagtcc tcatctccct caagcaggc 29

<210> SEQ ID NO 252
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 252

agctgcctgc ttgagggaga tgaggactg 29

<210> SEQ ID NO 253
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 253

ctagtctccc tcaagcaggc cccgctggt 29

<210> SEQ ID NO 254
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 254

agctaccagc ggggcctgct tgagggaga 29

<210> SEQ ID NO 255
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 255

ctagctgtga agagcttcac tgagtagga 29

<210> SEQ ID NO 256
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 256

agcttcctac tcagtgaagc tcttcacag 29

<210> SEQ ID NO 257
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 257

ctagtgcaga tgtagtgttt ccacagggt 29

<210> SEQ ID NO 258
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 258

agctaccctg tggaaacact acatctgca 29

<210> SEQ ID NO 259
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 259

gcgacacgga aatgttgaat actcat 26

<210> SEQ ID NO 260
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 260

ggagtcaggc aactatggat gaacg 25

<210> SEQ ID NO 261
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 261

actgtgaaga gcttcactga gtaggattaa gatattgcag atgtagtgtt tccacagggt 60

<210> SEQ ID NO 262
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 262

actgtgaaga gcttcactga gtaggattaa gatattgaag atgtagtgtt tccacagggt 60

<210> SEQ ID NO 263
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 263

actgtgaaga gcttcactga gtaggattaa gatattgaag atgtagtgtt tccactgggt 60

<210> SEQ ID NO 264
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 264

actgtgaaga gcttcactga gtaggattaa gatattgcag atggagggtt tccacagggt 60

<210> SEQ ID NO 265
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 265

actgtgaaga gcttcactga gtaggattaa gatattgcag atgtagtgtt accagagggt 60

<210> SEQ ID NO 266
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 266

actgtgaaga gcttcactga gtaggattaa gatattgggg atgtagtgtt tccactgggt 60

<210> SEQ ID NO 267
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 267

tccctcaagc aggccccgct ggtgcactga agagccaccc tgtggaaaca ctacatctgc 60

<210> SEQ ID NO 268
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 268

tccctcaagc aggccccgct ggtgcactga agagccaccc tgtggaaaca ctacatcttc 60

<210> SEQ ID NO 269
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 269

tccctcaagc aggccccgct ggtgcactga agagccaccc agtggaaaca ctacatcttc 60

<210> SEQ ID NO 270
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 270

tccctcaagc aggccccgct ggtgcactga agagccaccc tgtggaaacc ctccatctgc 60

<210> SEQ ID NO 271
<211> LENGTH: 60

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 271

tccctcaagc aggccccgct ggtgcactga agagccaccc tctggtaaca ctacatctgc 60

<210> SEQ ID NO 272
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 272

tccctcaagc aggccccgct ggtgcactga agagccaccc agtggaaaca ctacatcccc 60

<210> SEQ ID NO 273
<211> LENGTH: 62
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 273

aatgatacgg cgaccaccga gatctacact ctttccctac acgacgctct tccgatctaa 60

ca 62

<210> SEQ ID NO 274
<211> LENGTH: 49
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 274

tgttagatcg gaagagcgtc gtgtagggaa agagtgtaga tctcggtgg 49

<210> SEQ ID NO 275
<211> LENGTH: 62
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 275

aatgatacgg cgaccaccga gatctacact ctttccctac acgacgctct tccgatcttt 60

ca 62

<210> SEQ ID NO 276
<211> LENGTH: 49
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 276

tgaaagatcg gaagagcgtc gtgtagggaa agagtgtaga tctcggtgg 49

<210> SEQ ID NO 277
<211> LENGTH: 58
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:

<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 277

aatgatacgg cgaccaccga gatctacact ctttccctac acgacgctct tccgatct 58

<210> SEQ ID NO 278
<211> LENGTH: 45
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 278

agatcggaag agcgtcgtgt agggaaagag tgtagatctc ggtgg 45

<210> SEQ ID NO 279
<211> LENGTH: 15
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 279

gacggcatac gagat 15

<210> SEQ ID NO 280
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 280

aacaatctcg tatgccgtct tctgcttg 28

<210> SEQ ID NO 281
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 281

tcttatctcg tatgccgtct tctgcttg 28

<210> SEQ ID NO 282
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 282

agagatctcg tatgccgtct tctgcttg 28

<210> SEQ ID NO 283
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 283

ttgtatctcg tatgccgtct tctgcttg 28

<210> SEQ ID NO 284
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 284

caagcagaag acggcatacg agattgtgtt ctcggcaggt 40

<210> SEQ ID NO 285
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 285

caagcagaag acggcatacg agatagaaga ctcggcaggt 40

<210> SEQ ID NO 286
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 286

caagcagaag acggcatacg agatttctct ctcggcaggt 40

<210> SEQ ID NO 287
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 287

caagcagaag acggcatacg agatacacaa ctcggcaggt 40

<210> SEQ ID NO 288
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 288

aatgatacgg cgaccaccga 20

<210> SEQ ID NO 289
<211> LENGTH: 82
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (59)..(62)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 289

aatgatacgg cgaccaccga gatctacact ctttccctac acgacgctct tccgatctnn 60

nnacctacct gccgagaaca ca 82

<210> SEQ ID NO 290

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<211> LENGTH: 82
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (59)..(62)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 290

aatgatacgg cgaccaccga gatctacact ctttccctac acgacgctct tccgatctnn 60

nnacctacct gccgagtctt ct 82

<210> SEQ ID NO 291
<211> LENGTH: 82
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (59)..(62)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 291

aatgatacgg cgaccaccga gatctacact ctttccctac acgacgctct tccgatctnn 60

nnacctacct gccgagagag aa 82

<210> SEQ ID NO 292
<211> LENGTH: 82
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (59)..(62)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 292

aatgatacgg cgaccaccga gatctacact ctttccctac acgacgctct tccgatctnn 60

nnacctacct gccgagttgt gt 82

<210> SEQ ID NO 293
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 293

caagcagaag acggcatacg agat 24

<210> SEQ ID NO 294
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 294

acactctttc cctacacgac gctcttccga tctcaagtct agcaagcagg cca 53

<210> SEQ ID NO 295
<211> LENGTH: 53

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 295

acactctttc cctacacgac gctcttccga tctcaggcac tgagtgggaa agt 53

<210> SEQ ID NO 296
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 296

acactctttc cctacacgac gctcttccga tcttaacccc aagtcagcaa gca 53

<210> SEQ ID NO 297
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 297

acactctttc cctacacgac gctcttccga tctttgctgg tcaataccct ggc 53

<210> SEQ ID NO 298
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 298

acactctttc cctacacgac gctcttccga tcttgagtac ccctgaaatg ggc 53

<210> SEQ ID NO 299
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 299

acactctttc cctacacgac gctcttccga tcttcgctac caatcagggc ttt 53

<210> SEQ ID NO 300
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 300

acactctttc cctacacgac gctcttccga tctccattgc cacttgtttg cat 53

<210> SEQ ID NO 301
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 301

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acactctttc cctacacgac gctcttccga tctcctaccc ccacaacttt gct 53

<210> SEQ ID NO 302
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 302

acactctttc cctacacgac gctcttccga tctgtgtaca tccagtgcac cca 53

<210> SEQ ID NO 303
<211> LENGTH: 57
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 303

acactctttc cctacacgac gctcttccga tcttcggaaa ggactttgaa tacttgt 57

<210> SEQ ID NO 304
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 304

acactctttc cctacacgac gctcttccga tctcggccca agacctcatt cac 53

<210> SEQ ID NO 305
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 305

acactctttc cctacacgac gctcttccga tctgtcctct ctggggcaga agt 53

<210> SEQ ID NO 306
<211> LENGTH: 55
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 306

acactctttc cctacacgac gctcttccga tctagctgag tcatgagttg tctcc 55

<210> SEQ ID NO 307
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 307

acactctttc cctacacgac gctcttccga tctctgccag cttctcacac cat 53

<210> SEQ ID NO 308
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence

<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 308

acactctttc cctacacgac gctcttccga tctctgaagg acaaaggcgg gaa 53

<210> SEQ ID NO 309
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 309

acactctttc cctacacgac gctcttccga tctaaggtgc taaaggctcc acg 53

<210> SEQ ID NO 310
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 310

acactctttc cctacacgac gctcttccga tctgaccatt ggtgagccca gag 53

<210> SEQ ID NO 311
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 311

acactctttc cctacacgac gctcttccga tcttttttcg ggcaactgct cac 53

<210> SEQ ID NO 312
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 312

acactctttc cctacacgac gctcttccga tctgcaagcc ttctctcctc aga 53

<210> SEQ ID NO 313
<211> LENGTH: 54
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 313

acactctttc cctacacgac gctcttccga tctacacaaa cttccctgag accc 54

<210> SEQ ID NO 314
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 314

acactctttc cctacacgac gctcttccga tcttgagtta gccctgctgt tca 53

<210> SEQ ID NO 315
<211> LENGTH: 55
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 315

acactctttc cctacacgac gctcttccga tcttgaagag cttcactgag tagga 55

<210> SEQ ID NO 316
<211> LENGTH: 54
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 316

acactctttc cctacacgac gctcttccga tcttcccctt acagccaatt tcgt 54

<210> SEQ ID NO 317
<211> LENGTH: 58
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 317

acactctttc cctacacgac gctcttccga tcttgctgat gaaatgcaat taagaggt 58

<210> SEQ ID NO 318
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 318

acactctttc cctacacgac gctcttccga tctggtccct gcaagccagt atg 53

<210> SEQ ID NO 319
<211> LENGTH: 55
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 319

acactctttc cctacacgac gctcttccga tctatcaaag ccttgtatca cagtt 55

<210> SEQ ID NO 320
<211> LENGTH: 54
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 320

acactctttc cctacacgac gctcttccga tctcccaaat aatgcaggag ccaa 54

<210> SEQ ID NO 321
<211> LENGTH: 55
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 321

acactctttc cctacacgac gctcttccga tctctgcctt tagtgggaca gactt 55

<210> SEQ ID NO 322
<211> LENGTH: 54
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 322

acactctttc cctacacgac gctcttccga tctagtaacc ctagtagccc tcca 54

<210> SEQ ID NO 323
<211> LENGTH: 54
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 323

acactctttc cctacacgac gctcttccga tctcattgca gtgagccgag attg 54

<210> SEQ ID NO 324
<211> LENGTH: 54
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 324

acactctttc cctacacgac gctcttccga tcttggcaaa gttcacttcc atgt 54

<210> SEQ ID NO 325
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 325

acactctttc cctacacgac gctcttccga tcttgctctg tgatgtctgc cac 53

<210> SEQ ID NO 326
<211> LENGTH: 55
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 326

acactctttc cctacacgac gctcttccga tcttgtgtag gattgtgaac cagca 55

<210> SEQ ID NO 327
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 327

acactctttc cctacacgac gctcttccga tcttcccagc ccagcatttt tct 53

<210> SEQ ID NO 328

<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 328

acactctttc cctacacgac gctcttccga tctaggttgc tttgtgcaca gtc 53

<210> SEQ ID NO 329
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 329

acactctttc cctacacgac gctcttccga tctcctggct tgggatgttg gaa 53

<210> SEQ ID NO 330
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 330

acactctttc cctacacgac gctcttccga tctttgccca aggtcatact gct 53

<210> SEQ ID NO 331
<211> LENGTH: 56
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 331

acactctttc cctacacgac gctcttccga tctacccact aggtagccat aatcca 56

<210> SEQ ID NO 332
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 332

acactctttc cctacacgac gctcttccga tctcggtcat gtcgcttgga aga 53

<210> SEQ ID NO 333
<211> LENGTH: 56
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 333

acactctttc cctacacgac gctcttccga tctttggccc atattgcttt atgctg 56

<210> SEQ ID NO 334
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 334

acactctttc cctacacgac gctcttccga tctattaggg gttggctgca tga 53

<210> SEQ ID NO 335
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 335

acactctttc cctacacgac gctcttccga tctccaagac gtgttgcatg ctg 53

<210> SEQ ID NO 336
<211> LENGTH: 57
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 336

acactctttc cctacacgac gctcttccga tcttgggagg tgataaattc cctaaat 57

<210> SEQ ID NO 337
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 337

acactctttc cctacacgac gctcttccga tctccagaga caaaggtggg gag 53

<210> SEQ ID NO 338
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 338

acactctttc cctacacgac gctcttccga tcttcataca gaagagcaaa gtacca 56

<210> SEQ ID NO 339
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 339

acactctttc cctacacgac gctcttccga tctcaaagag gggtatcggg agc 53

<210> SEQ ID NO 340
<211> LENGTH: 57
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 340

acactctttc cctacacgac gctcttccga tctaaatgga agaaccaagt agatgaa 57

<210> SEQ ID NO 341
<211> LENGTH: 55
<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 341

acactctttc cctacacgac gctcttccga tctttttggt tgacagatgg ccaca 55

<210> SEQ ID NO 342
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 342

acactctttc cctacacgac gctcttccga tcttcttact tgtgtgattt tagaacaa 58

<210> SEQ ID NO 343
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 343

acactctttc cctacacgac gctcttccga tctgatggtt catgcagagg gct 53

<210> SEQ ID NO 344
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 344

acactctttc cctacacgac gctcttccga tctgctggtc tttcctgagc tgt 53

<210> SEQ ID NO 345
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 345

acactctttc cctacacgac gctcttccga tctctccatc agatacctgt accca 55

<210> SEQ ID NO 346
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 346

acactctttc cctacacgac gctcttccga tctgggaaaa cactctctct ctgct 55

<210> SEQ ID NO 347
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 347

acactctttc cctacacgac gctcttccga tctggaggcc acgacacaca ata 53

<210> SEQ ID NO 348
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 348

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<210> SEQ ID NO 349
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 349

gtgactggag ttcagacgtg tgctcttccg atcttgcaca tgtttccaca gggt 54

<210> SEQ ID NO 350
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 350

gtgactggag ttcagacgtg tgctcttccg atctagtgtt tccaggagcg gttt 54

<210> SEQ ID NO 351
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 351

gtgactggag ttcagacgtg tgctcttccg atctaagcct caggcacaac tctg 54

<210> SEQ ID NO 352
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<212> TYPE: DNA
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 352

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<210> SEQ ID NO 353
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<212> TYPE: DNA
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 353

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<212> TYPE: DNA
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 354

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<210> SEQ ID NO 355
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 355

gtgactggag ttcagacgtg tgctcttccg atcttcactg cctgggtgct ttag 54

<210> SEQ ID NO 356
<211> LENGTH: 54
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 356

gtgactggag ttcagacgtg tgctcttccg atcttacccc agcctccagc ttta 54

<210> SEQ ID NO 357
<211> LENGTH: 54
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 357

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<210> SEQ ID NO 358
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 358

gtgactggag ttcagacgtg tgctcttccg atctaggctg ttatgcagga aaggaa 56

<210> SEQ ID NO 359
<211> LENGTH: 54
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 359

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<210> SEQ ID NO 360
<211> LENGTH: 54
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 360

gtgactggag ttcagacgtg tgctcttccg atctggcagc atcccttaca tcct 54

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<210> SEQ ID NO 361
<211> LENGTH: 57
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 361

gtgactggag ttcagacgtg tgctcttccg atctagaaaa agcttcccca gaaagga 57

<210> SEQ ID NO 362
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 362

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<210> SEQ ID NO 363
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 363

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<210> SEQ ID NO 364
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 364

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<210> SEQ ID NO 365
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 365

gtgactggag ttcagacgtg tgctcttccg atctttccta gcgggaactg gaaa 54

<210> SEQ ID NO 366
<211> LENGTH: 54
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 366

gtgactggag ttcagacgtg tgctcttccg atctaggcta atggggtagg ggat 54

<210> SEQ ID NO 367
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 367

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<210> SEQ ID NO 368
<211> LENGTH: 54
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 368

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<210> SEQ ID NO 369
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 369

gtgactggag ttcagacgtg tgctcttccg atctagcagg ccaaagatgt ctcc 54

<210> SEQ ID NO 370
<211> LENGTH: 57
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 370

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<210> SEQ ID NO 371
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 371

gtgactggag ttcagacgtg tgctcttccg atctgggacc aatttgctac tcatgg 56

<210> SEQ ID NO 372
<211> LENGTH: 54
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 372

gtgactggag ttcagacgtg tgctcttccg atcttggagg ctgtaaacgt cctg 54

<210> SEQ ID NO 373
<211> LENGTH: 59
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 373

gtgactggag ttcagacgtg tgctcttccg atcttgctat gatttgctga attactcct 59

<210> SEQ ID NO 374
<211> LENGTH: 55

<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 374

gtgactggag ttcagacgtg tgctcttccg atctgcaatt ttgcagacca ccatc 55

<210> SEQ ID NO 375
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 375

gtgactggag ttcagacgtg tgctcttccg atctggcagc ttgcaacctt cttg 54

<210> SEQ ID NO 376
<211> LENGTH: 54
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 376

gtgactggag ttcagacgtg tgctcttccg atcttcatga gagtttcccc aaca 54

<210> SEQ ID NO 377
<211> LENGTH: 58
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 377

gtgactggag ttcagacgtg tgctcttccg atctacttga gggggaaaaa gtttctta 58

<210> SEQ ID NO 378
<211> LENGTH: 54
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 378

gtgactggag ttcagacgtg tgctcttccg atcttggtcc ctgtctgtca ttgg 54

<210> SEQ ID NO 379
<211> LENGTH: 54
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 379

gtgactggag ttcagacgtg tgctcttccg atctaagcga gtgactgtct ggga 54

<210> SEQ ID NO 380
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 380

gtgactggag ttcagacgtg tgctcttccg atctcatggg tgggacacgt agtt 54

<210> SEQ ID NO 381
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 381

gtgactggag ttcagacgtg tgctcttccg atctggcttt cctggacacc ctatc 55

<210> SEQ ID NO 382
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 382

gtgactggag ttcagacgtg tgctcttccg atctagagcg agggagcgat gta 53

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 383

gtgactggag ttcagacgtg tgctcttccg atctttgtgg accactgctt agtgc 55

<210> SEQ ID NO 384
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 384

gtgactggag ttcagacgtg tgctcttccg atctcaacta ccctgaggcc acc 53

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 385

gtgactggag ttcagacgtg tgctcttccg atctggtcag cactcctcag cttt 54

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 386

gtgactggag ttcagacgtg tgctcttccg atcttggagg atgcatgcca catt 54

<210> SEQ ID NO 387
<211> LENGTH: 54
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence

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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 387

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<210> SEQ ID NO 388
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 388

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<210> SEQ ID NO 389
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 389

gtgactggag ttcagacgtg tgctcttccg atcttagata tatgggtgtg tctgtacg 58

<210> SEQ ID NO 390
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 390

gtgactggag ttcagacgtg tgctcttccg atctttccaa agtggctgaa ccat 54

<210> SEQ ID NO 391
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<220> FEATURE:
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<400> SEQUENCE: 391

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<210> SEQ ID NO 392
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 392

gtgactggag ttcagacgtg tgctcttccg atctttgtaa tgcaacctct gtcatgc 57

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<212> TYPE: DNA
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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 393

gtgactggag ttcagacgtg tgctcttccg atctccagct ccagcaatcc atga 54

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<400> SEQUENCE: 394

gtgactggag ttcagacgtg tgctcttccg atcttttggg aaagatagcc ctgga 55

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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 395

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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 396

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<210> SEQ ID NO 397
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 397

gtgactggag ttcagacgtg tgctcttccg atctcagatc cctcctgggc aatg 54

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<212> TYPE: DNA
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<400> SEQUENCE: 398

gtgactggag ttcagacgtg tgctcttccg atctgtcagg aggcaaggag gaac 54

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<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 399

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<400> SEQUENCE: 400

gtgactggag ttcagacgtg tgctcttccg atctgcggca gattcctggt gatt 54

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 401

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<210> SEQ ID NO 402
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 402

caagcagaag acggcatacg agatatatca gtgtgactgg agttcagacg tgtgct 56

<210> SEQ ID NO 403
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 403

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<210> SEQ ID NO 404
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 404

caagcagaag acggcatacg agatccactc atgtgactgg agttcagacg tgtgct 56

<210> SEQ ID NO 405
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 405

caagcagaag acggcatacg agattacgta cggtgactgg agttcagacg tgtgct 56

<210> SEQ ID NO 406
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 406

caagcagaag acggcatacg agatcgaaac tcgtgactgg agttcagacg tgtgct 56

<210> SEQ ID NO 407

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<211> LENGTH: 56
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 407

caagcagaag acggcatacg agatatcagt atgtgactgg agttcagacg tgtgct 56

<210> SEQ ID NO 408
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 408

aatgatacgg cgaccaccga gatctacaca ttactcgaca ctctttccct acacgac 57

<210> SEQ ID NO 409
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 409

aatgatacgg cgaccaccga gatctacact ccggagaaca ctctttccct acacgac 57

<210> SEQ ID NO 410
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Oligonucleotide

<400> SEQUENCE: 410

aatgatacgg cgaccaccga gatctacacc gctcattaca ctctttccct acacgac 57

What is claimed is:

1. A method for identifying a target site of an RNA-programmable nuclease, the method comprising

(a) providing an RNA-programmable nuclease that cuts a double-stranded nucleic acid target site, wherein cutting of the target site results in cut nucleic acid strands comprising a 5' phosphate moiety;

(b) contacting the RNA-programmable nuclease of (a) with a library of candidate nucleic acid molecules, wherein each nucleic acid molecule comprises a concatemer of a sequence comprising a candidate nuclease target site and a constant insert sequence of at least 10 and not more than 70 nucleotides, under conditions suitable for the RNA-programmable nuclease to cut a candidate nucleic acid molecule comprising a target site of the RNA-programmable nuclease; and

(c) identifying nuclease target sites cut by the RNA-programmable nuclease in (b) by direct sequencing of an intact nuclease target site on the nucleic acid strand that was cut by the RNA-programmable nuclease in step (b), wherein the method does not include computational reconstruction of nuclease target sites from cut half-sites.

2. The method of claim **1**, wherein the method identifies off-target sites of the RNA-programmable nuclease.

3. The method of claim **1**, wherein the RNA-programmable nuclease creates blunt ends.

4. The method of claim **1**, wherein of step (c) comprises ligating a first nucleic acid adapter to the 5' end of a nucleic acid strand that was cut by the RNA-programmable nuclease in step (b) via 5'-phosphate-dependent ligation.

5. The method of claim **4**, wherein the nucleic acid adapter is provided in double-stranded form.

6. The method of claim **5**, wherein the 5'-phosphate-dependent ligation is a blunt end ligation.

7. The method of claim **4**, wherein step (c) comprises amplifying a fragment of the concatemer cut by the RNA-programmable nuclease that comprises an uncut target site via a PCR reaction using a PCR primer that hybridizes with the adapter and a PCR primer that hybridizes with the constant insert sequence.

8. The method of claim **7**, wherein the method further comprises enriching the amplified nucleic acid molecules for molecules comprising a single uncut target sequence.

9. The method of claim **1**, wherein step (c) comprises sequencing the nucleic acid strand that was cut by the nuclease in step (b), or a copy thereof obtained via PCR, using a high-throughput sequencing method.

10. The method of claim **1**, wherein the library of candidate nucleic acid molecules comprises at least 10^{10}, different candidate nuclease cleavage sites.

11. The method of claim **1**, wherein the nuclease is a therapeutic nuclease which cuts a specific nuclease target site in a gene associated with a disease.

12. The method of claim 11, further comprising determining a maximum concentration of the therapeutic nuclease at which the therapeutic nuclease cuts the specific nuclease target site, and does not an additional nuclease target site.

13. The method of claim 12, further comprising administering the therapeutic nuclease to a subject in an amount effective to generate a final concentration equal or lower than the maximum concentration.

14. The method of claim 1, wherein the nuclease is an RNA-programmable nuclease that forms a complex with an RNA molecule, and wherein the nuclease:RNA complex specifically binds a nucleic acid sequence complementary to the sequence of the RNA molecule.

15. The method of claim 14, wherein the RNA molecule is a single-guide RNA (sgRNA).

16. The method of claim 14, wherein the RNA molecule comprises 15-25 nucleotides that are complementary to the target sequence.

17. The method of claim 1, wherein the nuclease is a Cas9 nuclease.

18. The method of claim 14, wherein the nuclease target site comprises a [sgRNA-complementary sequence]-[protospacer adjacent motif (PAM)] structure, and the nuclease cuts the target site within the sgRNA-complementary sequence.

19. The method of claim 18, wherein the sgRNA-complementary sequence comprises 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides.

20. The method of claim 1, wherein step (c) comprises identifying nuclease target sites cut by the RNA-programmable nuclease by direct, single-end sequencing of an intact nuclease target site on the nucleic acid strand that was cut by the RNA-programmable nuclease.

21. The method of claim 1, wherein both a cut half-site and an adjacent uncut nuclease target site of the same library member are contained within a 100 nucleotide sequence.

22. The method of claim 1, wherein the constant insert sequence comprises not more than 60 nucleotides.

23. The method of claim 1, wherein the constant insert sequence comprises not more than 55 nucleotides.

24. The method of claim 1, wherein the library of candidate nucleic acid molecules comprises at least 10^{12} different candidate nuclease cleavage sites.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 9,163,284 B2
APPLICATION NO. : 14/320370
DATED : October 20, 2015
INVENTOR(S) : Liu et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the Claims

Claim 4, column 238, line 41:

"wherein of step (c)" should be changed to --wherein step (c)--.

Claim 10, column 238, line 63:

"at least 10^{10}, different" should be changed to --at least 10^{10} different--.

Signed and Sealed this
Fourteenth Day of June, 2016

Michelle K. Lee

Michelle K. Lee
Director of the United States Patent and Trademark Office